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BIOMARCADORES NO CÂNCER CERVICAL: ASPECTOS MOLECULARES E COMPUTACIONAIS

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Recife, 2017

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Tese de doutorado apresentada ao Programa de Pós-Graduação em Biologia Aplicada à Saúde, da Universidade Federal de Pernambuco, como requisito para a obtenção do título de Doutora em Biologia Aplicada à Saúde.

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RESUMO

O câncer cervical é a quarta neoplasia mais frequente em mulheres no mundo, no qual as pacientes são submetidos a tratamentos quimioterápicos que levam à grande desgaste fisiológico e até mesmo remoção do útero. O presente trabalho teve como objetivo identificar marcadores moleculares que possam contribuir no diagnóstico precoce e prognóstico, além de auxiliar na conduta terapêutica em pacientes acometidas por esta neoplasia. Desta forma, foi realizada análise do padrão de expressão moléculas relacionadas à infecção por HPV (papiloma vírus humano) através de qPCR em amostras de lesão e câncer cervical. Amostras de raspado cervical mostraram diminuição da expressão de IL-10 (interleucina 10) entre pacientes positivos para infecção por HPV e amostras de câncer cervical (p=0.0005). Verificou-se expressão aumentada de SIRT5 (sirtuina 5) em câncer cervical relativamente à expressão de IL-10 (p<0.0001) e SIRT1 (p=0.0014) que pode levar à mitigação da hipóxia induzida por infecção de HPV através da neutralização de ROS (espécies reativas de oxigénio). Foram realizadas análises computacionais visando a seleção de genes com potencial para biomarcador no câncer de cervical e a identificação do efeito das mutações em moléculas de potencial terapêutico. A seleção de genes com alterações tanto a nível de DNA (variantes e metilação) quanto à nível de expressão de mRNA/proteína em câncer cervical revelou 4 marcadores que poderiam auxiliar no diagnóstico e prognóstico da doença: CDH1 (e-caderina), CDKN2A (inibidor ciclina quinase dependente 2A), RB1 (retinoblastoma) e TP53 (supressor de tumor 53). Foram avaliados os impactos funcionais, estruturais de mutações não-sinônimas e variação de estabilidade nas moléculas VEGF (fator de crescimento vascular endotelial), VEGFR-2 (receptor do fator de crescimento vascular endotelial 2) e EGFR (receptor do fator de crescimento epidérmico) reportadas como afetando o uso de diferentes terapias em câncer. O uso da base de dados dbNSFP demonstrou que EGFR L858R pode ser um bom indicador terapêutico, uma vez que leva à maior sensibilidade aos inibidores tirosina-quinase; enquanto EGFR K745T não apresenta atividade catalítica, sendo um bom marcador para direcionar a terapia alvo de escolha. Tais dados podem contribuir para o desenvolvimento de painéis moleculares comerciais voltados para pacientes de câncer de cervical, assim como auxiliar no direcionamento terapêutico.

Palavas-Chave: Câncer cervical. Marcadores moleculares. Bioinformática. Algoritmos.

ABSTRACT

Cervical cancer is the fourth most frequent neoplasia worldwide. Cervical cancer patients undergo chemotherapy, which leads to high physiological stress and even uterus removal. This work goal was to identify molecular markers that could aid in early diagnosis and prognosis assessment and contribute to target therapy in cervical cancer patients. It was performed expression pattern analysis of molecules related to HPV (human papillomavirus) infection through qPCR in lesion and cervical cancer samples. Cervix smear samples showed lower expression of IL-10 (interleukin 10) between HPV^+ and cervical cancer samples (p=0.0005). It was observed higher expression of SIRT5 (sirtuin 5) in cervical cancer compared to IL-10 (p<0.0001) and SIRT1(p=0.0014) which could lead to mitigate the hypoxia induced by HPV infection through ROS (reactive oxygen species) neutralization. It was performed computational analysis to select genes that have biomaker potential in cervical cancer and identification of molecules with therapeutic potential. The selected genes had DNA alterations (variants and methylation) and mRNA/protein expression. In cervical cancer showed four markers that could aid in disease diagnisis and prognosis CDKN2A (ciclin dependent kinase inhibitor 2A), CDH1 (E-cadherin), RB1 (retinoblastoma) and TP53 (tuor suppressor 53). In relation to the target therapy in cervical cancer it were assessed the functional and structural impacts of non-synonimous mutations and stability alteration in VEGF (vascular endotelial growth fator), VEGFR-2 (receptor of vascular endotelial growth fator 2) and EGFR (epidermal growth fator receptor) reported to have impact in cancer therapies. The use of dbNSFP showed that EGFR L858R could be a good therapeutic indicator as is leads to higher sensibility to tyrosine kinase inhibitors; wereas EGFR K745Rdoes not have catalytic activity, being a good marker to target therapy. Such data could contribute to the development of comercial molecular panels towards cervical cancer patients as well as aid in therapeutic targeting.

Keywords: Cervical cancer. Molecular markers. Bioinformatics. Algorithms.

SUMÁRIO

1 INTRODUÇÃO

O câncer cervical é um importante problema de saúde pública no mundo devido à sua alta incidência (mais de 528.000 casos em 2012) e prevalência a cinco anos de 1.547.000 casos, estando em quarto lugar entre os tumores femininos mais frequentes, chegando a 7,4% dos casos mundiais de câncer.

A causa necessária para o desenvolvimento de câncer cervical é a infecção pelo papilomavírus humano (HPV). A infecção por HPVs de alto risco leva à integração do DNA viral no hospedeiro e consequente expressão das proteínas virais E6 e E7, que são responsáveis por levar à inativação de importantes supressores tumorais, levando dessa forma à fuga da apoptose, um dos marcos para o desenvolvimento do câncer. O rastreio populacional através de exames periódicos de citologia oncótica e a vacinação têm sido as opções profiláticas. Pacientes acometidos pelo câncer cervical são submetidos a tratamentos quimioterápicos, que levam à grande desgaste fisiológico e até mesmo remoção do útero, causando um grande impacto psicológico, especialmente em pacientes jovens.

O advento das tecnologias moleculares levou à descoberta de moléculas biomarcadoras, podendo ser de caráter de diagnóstico, prognóstico ou mesmo preditor da resposta terapêutica. Hoje em dia já existem biomarcadores para diversos tipos de câncer como o receptor tirosina quinase erb-b2 (HER2) em câncer de mama e mutações específicas no receptor de fator de crescimento epidérmico (EGFR) em câncer de pulmão, ambos permitindo um melhor direcionamento terapêutico. No entanto, o câncer cervical não apresenta biomarcadores específicos para direcionamento terapêutico adequado ou uma melhor avaliação de prognóstico.

Nos últimos anos, aliados a trabalhos experimentais, tem sido cada vez mais usadas ferramentas computacionais para auxiliar na interpretação e previsão de processos biológicos. Estas ferramentas, que operam na interface entre a biologia e informática, são de grande importância pois permitem prever as consequências biológicas de determinada característica, numa fração do tempo e custo. Existem diversos tipos de algoritmos usados em bioinformática, permitindo analisar impactos de mutações a nível funcional, estrutural e de estabilidade; fazer estudos de acoplamento para verificar a ligação de anticorpos/moléculas alvo, entre várias outras potencialidades.

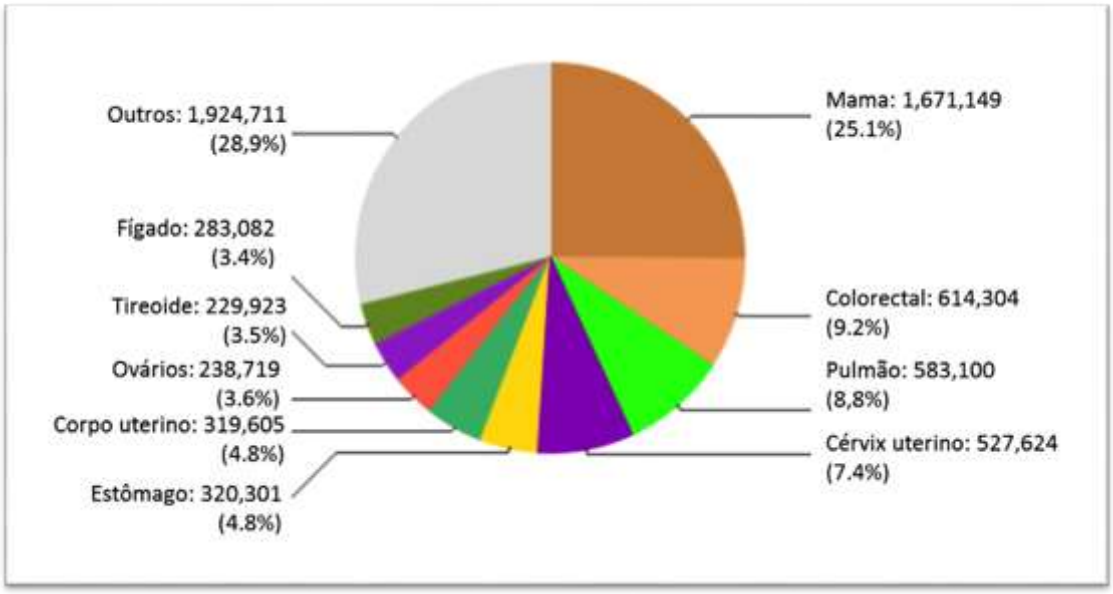
Desta forma, o presente trabalho visa contribuir no diagnóstico precoce, no prognóstico e na conduta terapêutica baseada em alvos moleculares como forma de aumentar a qualidade de vida de pacientes com câncer do colo do útero.

2 REFERENCIAL TEÓRICO

2.1 EPIDEMIOLOGIA DO CÂNCER

Segundo o Globocan, em 2012 existiam 14,1 milhões de novos casos de câncer a nível mundial, culminando em 8,2 milhões de mortes. Também se verificou que 32,6 milhões de pessoas viviam com câncer (diagnóstico em até 5 anos). Mundialmente, o câncer do colo uterino é o quarto mais incidente em mulheres (Figura 1) contribuindo com mais de 527 mil novos casos em 2012 representando 7,4% do total de casos (FERLAY et al., 2012).

Figura 1 – Incidência mundial de câncer em mulheres.



Fonte: adaptado de FERLAY e colab., 2012.

De uma forma geral, tanto a incidência quanto a mortalidades causadas pelo câncer cervical são maiores em países subdesenvolvidos; Ásia e África juntos representam 72,9% e 76,9% da incidência e mortalidade associadas com câncer cervical a nível mundial, respectivamente.

As previsões do INCA para 2016 foram de 16.340 novos casos de câncer cervical apresentando risco estimado de 15,85 novos casos a cada 100 mil mulheres. No estado de Pernambuco foi estimado o surgimento de 970 novos casos, dos quais 150

foram previstos para a capital do estado – Recife (INSTITUTO NACIONAL DE CANCER JOSÉ ALENCAR GOMES DA SILVA, 2016).

2.2 CÂNCER CERVICAL

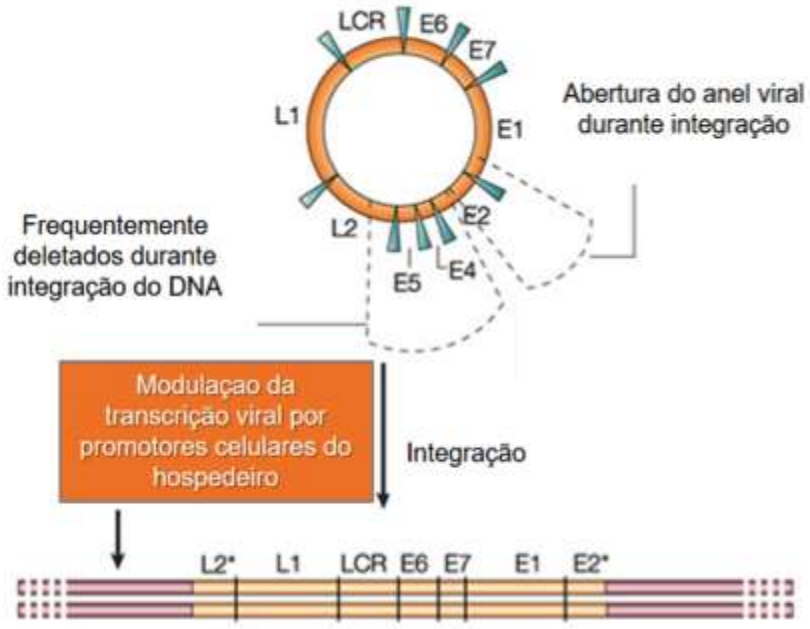
O câncer cervical ou câncer do colo uterino se desenvolve a partir de células infectadas por papiloma vírus humano (HPV) de alto risco (em 99% dos casos), e tipicamente se origina na junção escamo-colunar, uma área de elevada divisão celular. A conexão entre a infecção por infecção de HPV e desenvolvimento de câncer cervical foi descoberta em 1984 por Dr. Harald zur Hausen, que ganhou prémio Nobel pelo isolamento dos HPV-16 e -18 (ANGIOLI et al., 2016; BOSHART et al., 1984).

Já foram identificados cerca de 100 tipos de HPV e pelo menos 40 deles podem infectar a área genital. Estima-se que entre 50 a 80% das mulheres sexualmente ativas é infectada com pelo menos um tipo de HPV durante a sua vida, contudo a maioria das infecções por HPV são limitadas e assintomáticas ou não são detectadas. A infecção com HPV de alto risco ou oncogênico (principalmente os HPV-16 e -18) leva ao desenvolvimento de cerca de 99% dos pré-cânceres cervicais, enquanto os HPV de baixo risco ou não oncogênicos (como o HPV-6 e -11) causam verrugas genitais. A infecção persistente com HPV de alto risco é o maior fator de risco associado ao desenvolvimento de câncer cervical (ANGIOLI et al., 2016; WORKOWSKI; BOLAN, 2015).

O HPV precisa infectar as células do epitélio basal localizadas na zona de transformação cervical, que se replicam e diferenciam ativamente, para estabelecer a infecção. O genoma do HPV (Figura 2) é composto por proteínas precoces (E1, E2, E4, E5, E6 e E7) e proteínas estruturais tardias (L1 e L2) (ANGIOLI et al., 2016; WOODMAN et al., 2007). As proteínas E6 e E7 são duas unidades transcricionais que desempenham papel causal no desenvolvimento de câncer cervical. E6 promove a degradação de p53 (geralmente através de ubiquitinação) e E7 que inativa a proteína de retinoblastoma (pRb). No estado hipofosforilado, as proteínas da família pRb conseguem se ligar a fatores de transcrição como o E2F e reprimir a expressão de genes envolvidos em síntese de DNA e progressão de ciclo celular. E7 possui capacidade de

se ligar a pRb, o que leva as células a entrarem na fase S prematuramente através da quebra do complexo pRb-E2F (MOODY; LAIMINS, 2010).

Figura 2 - Organização do genoma do HPV e a sua integração nas células do hospedeiro.



Fonte: adaptado de ZUR HAUSEN e colab., 2002.

Relativamente à oncoproteína E6, ela é capaz de aumentar a indução de fator indutor de hipóxia (HIF) 1 alfa, o que consequentemente leva a aumento da expressão de fator de crescimento vascular endotelial (VEGF), o que faz com que seja um alvo terapêutico válido para câncer cervical. Além dos papéis determinantes de E6 e E7 na transformação maligna, a oncoproteína E5 ganhou mais interesse devido à sua implicação na carcinogênese cervical.

E5 pode contribuir para a carcinogênese cervical por diversos mecanismos, incluindo a ativação da via do receptor do fator de crescimento epidérmico (EGFR), modulação de vias de sinalização inflamatórias, indução da angiogênese através do VEGF e inibição da apoptose (MENDERES et al., 2015; MOODY; LAIMINS, 2010).

Independentemente do tipo, partículas infecciosas virais chegam as células germinativas na camada basal presumivelmente através de micro-traumas na mucosa (SCHIFFMAN et al., 2007). A relação sexual com penetração não se faz estritamente

necessária para transmissão do vírus, e tipos de HPV podem ser transferidos para o cérvix a partir da infecção inicial na entrada vaginal (WINER et al., 2003). Cerca de um terço das mulheres que possuem níveis de infecção por HPV detectados por teste de DNA têm anomalias citopatólogicas detectadas, uma vez que as alterações citológicas são menos sensíveis para detecção de HPV que testes moleculares. As lesões intraepiteliais escamosas de alto grau (LIEAG) são mais susceptíveis de serem causadas por infecção HPV-16 e tipos relacionados; por outro lado, infecção por HPV-18 está associado a um nível baixo deste tipo de lesões (KOVACIC et al., 2006). Este fato pode explicar, ao menos parcialmente, a baixa eficiência de triagem para lesões endocervicais e o aumento da proporção de adenocarcinoma, que apresenta associação à infecção por HPV-18 (BERRINGTON DE GONZÁLEZ; GREEN, 2007).

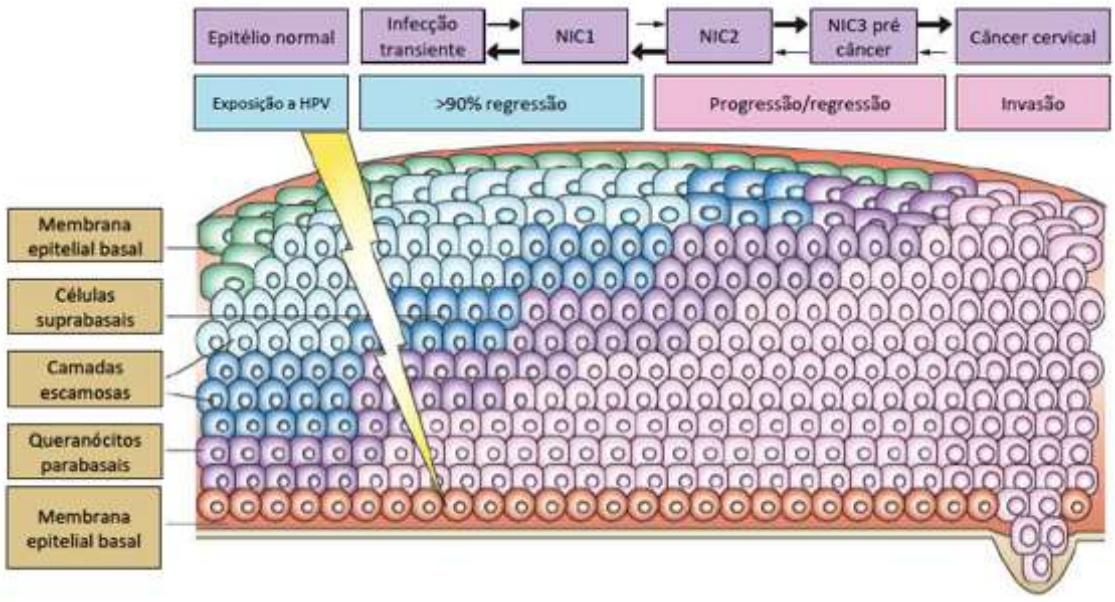
A infecção por HPV pode levar à anomalias citológicas ou histológicas, maioritariamente Neoplasia Cervical Intraepitelial 1 (NIC1), normalmente não-tratado devido à sua alta taxa de regressão. Lesões do tipo NIC2 são heterogêneas, podendo ser produzidas por tipos de HPV não-carcinogênicos e, portanto, não se trata de pré-câncer (CASTLE et al., 2007). No entanto, quando infecções por HPV carcinogênico persistem, lesões NIC3 podem surgir devido à instabilidade genética e expansão clonal de células altamente transformadas. Em termos histopatológicos, pré-câncer caracteriza-se pelo diagnóstico morfológico de NIC3, displasia severa ou carcinoma *in situ*. No estágio pré-câncer, células indiferenciadas com anomalias constituem quase a totalidade da espessura do epitélio cervical (VON KNEBEL DOEBERITZ, 2002).

A remoção de infecção por HPV na zona de transformação cervical pode ocorrer relativamente rápido através da imunidade inata e adaptativa ou outros mecanismos ainda não definidos. A maioria das infecções cervicais por HPV são suprimidas por imunidade mediada por células em um período de 6 meses a 2 anos após a exposição (PLUMMER et al., 2007; STANLEY, 2006). Uma pequena proporção (cerca de 10%) das infecções carcinogênicas persistem por vários anos, estando fortemente associadas com risco elevado de diagnóstico de pré-câncer (SCHIFFMAN et al., 2005). Ainda não está claro se as infecções regridem por completa remoção viral ou pela manutenção em um estado latente no epitélio basal, nos quais os vírus se replicam em níveis baixos sem expressão viral completa (STRICKLER et al., 2005).

No entanto, a infecção por HPV leva à redução de apoptose celular e ao crescimento celular desregulado (LEES; ERICKSON; HUH, 2016). Desta forma, uma infecção persistente por HPV pode levar ao desenvolvimento de câncer principalmente

em zonas de transformação entre diferentes tipos de epitélio, como por exemplo o cérvix uterino (SCHIFFMAN et al., 2007). O risco de desenvolvimento de câncer cervical está maioritariamente associado à infecção por HPV, no entanto outros fatores de risco podem influenciar no desenvolvimento da lesão, tais como: tabagismo, multiparidade, uso de contraceptivos orais por longos períodos que podem acarretar até três vezes maior risco de desenvolvimento de câncer cervical em mulheres infectadas com HPV carcinogênico, idade, mutagênicos, entre outros (APPLEBY et al., 2006; RAJKUMAR et al., 2006; SMITH et al., 2003; WHEELER, 2007). A carcinogênese cervical é induzida por HPV em 99% dos casos e a integração do DNA viral no genoma da célula do hospedeiro é frequentemente detectada tanto em lesões de alto grau como em câncer invasivo. O desenvolvimento do câncer cervical pode ser resumido em quatro passos: transmissão de HPV, persistência viral, progressão de um clone infectado persistentemente para pré-câncer e invasão. No entanto, a infecção pode desaparecer ou pode ocorrer regressão de lesão pré-cancerígena à normalidade (Figura 3) (SCHIFFMAN et al., 2007).

Figura 3 - Representação esquemático dos eventos decorrentes da infeção por HPV.



Fonte: adaptado de WHEELER e colab., 2007.

A prevenção de câncer cervical começou sendo feita através de exame Papanicolaou e atualmente inclui processos de análise citológica, teste de papiloma

vírus de alto risco, colposcopia, dentre outros (LEES; ERICKSON; HUH, 2016). Além disso, a vacinação contra o HPV é a forma primária de prevenção da infecção (LOPEZ MS et al., 2017).

Foram criadas vacinas profiláticas contra alguns genótipos de HPV. A primeira vacina criada foi aprovada em 2006 pela *Food and Drug Administration* nos Estados Unidos, consistindo na partícula do tipo viral L1 produzida de forma recombinante para quatro tipos de HPV: -6, -11, -16 e -18. Entretanto surgiu uma vacina divalente em 2009 específica apenas para os HPV-16 e HPV-18. Ambas as vacinas permitem o estímulo de resposta neutralizadora contra o HPV através de anticorpos, fornecendo proteção contra lesões pré-cancerígenas. Contudo, não apresentam a eficácia desejada em mulheres já infectadas por HPV e em mulheres com histórico de infecção com outros genótipos de HPV que não sejam contemplados pelas vacinas (BAVA; THULASIDASAN; SREEKANTH, 2016; SCHIFFMAN et al., 2007).

O tratamento contra o câncer cervical é feito de forma específica para o estadiamento do câncer. Na América Latina o tratamento primário para estadiamento precoce passa por histerectomia radical com linfoadenectomia pélvica levando à diminuição da qualidade de vida e remoção de fertilidade. Devido a isso têm surgido a necessidade de técnicas cirúrgicas menos radicais para as mulheres como por exemplo a conização (KANG et al., 2015; LOPEZ MS et al., 2017; LORUSSO et al., 2014). Nas pacientes com doença localmente avançada a opção terapêutica passa pela associação de quimioterapia com radioterapia. O tratamento radioterápico decorre em cinco dias por semana num período de cinco ou seis semanas, seguido por dois a cinco tratamentos de braquiterapia. Concomitantemente, a quimioterapia com cisplatina é aplicada uma vez por semana durante o período do tratamento (LOPEZ MS et al., 2017)

Atualmente, a histerectomia não é mais uma opção terapêutica primária viável para lesões pré-cancerígenas e usam-se outros métodos. A conização e crioterapia são as primeiras opções usadas em lesões intraepiteliais do colo uterino, não comprometendo a fertilidade da paciente (SCHIFFMAN et al., 2007).

2.3 BIOMARCADORES NO CÂNCER

Em 1998 o National Institutes of Health Biomarkers Definitions Working Group definiu um biomarcador como sendo "uma característica que pode ser medida de forma objetiva e avaliada como indicador de processos biológicos normais, patogênicos ou respostas farmacológicas a intervenção terapêutica" (STRIMBU; TAVEL, 2011).

Estes biomarcadores podem existir a 3 níveis: DNA, RNA e proteína. Dentre os biomarcadores em DNA existem os polimorfismos de base única (SNP), aberrações cromossômicas, alterações no número de cópias de DNA, e metilação da região promotora do gene. No caso de marcadores com base em RNA, eles incluem aumento ou diminuição da expressão do gene, e microRNAs. Quanto aos biomarcadores protéicos, eles incluem receptores de superfície, antígenos tumorais, estados de fosforilação (LUDWIG; WEINSTEIN, 2005).

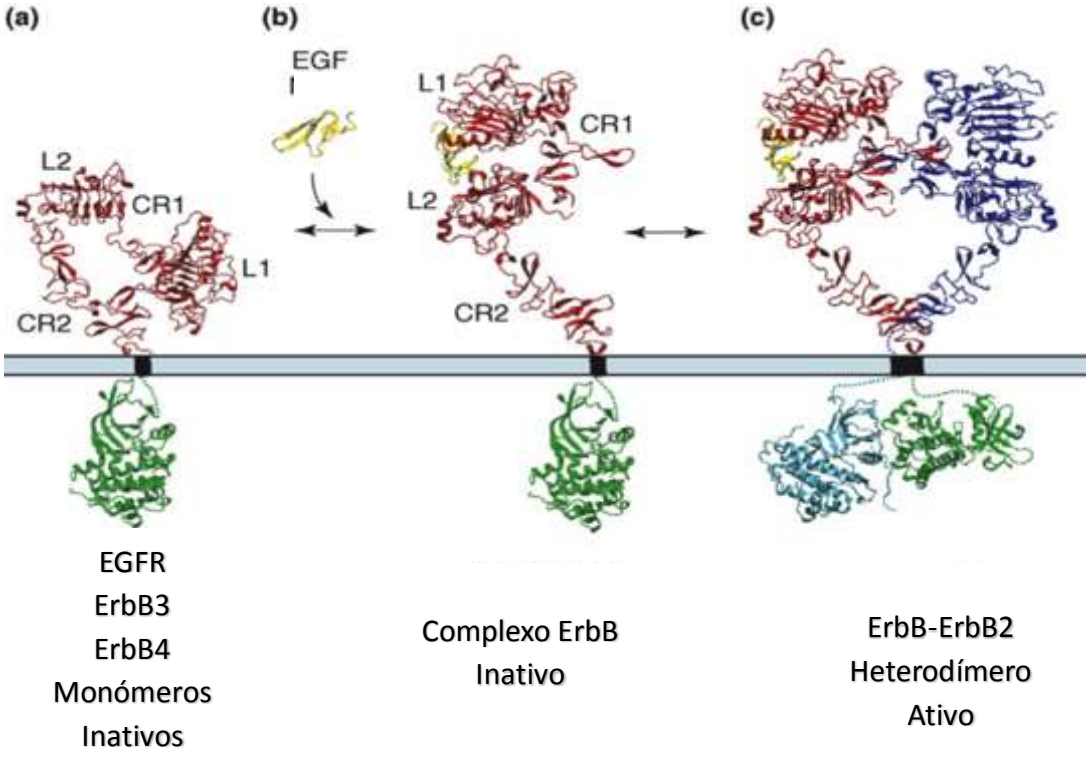
Existem diversos biomarcadores específicos já estabelecidos para outros tipos de câncer; no câncer de mama, a expressão do receptor de estrógeno e receptor HER2 é um indicador de melhor prognóstico, assim como permite um melhor direcionamento terapêutico a essas pacientes (LUDWIG; WEINSTEIN, 2005). Além disso, em câncer de pulmão de células não-pequenas (CPCNP), as variantes de EGFR podem levar a um melhor direcionamento terapêutico (PENG; SONG; JIAO, 2015).

2.3.1 Receptor do Fator de Crescimento Epidérmico (EGFR)

A família de fatores de crescimento epidérmicos é constituída por quatro membros: EGFR/HER1/ErbB1, HER2/ErbB2, HER3/ErbB3 e HER4/ErbB4 os quais são estruturalmente semelhantes (CAPDEVILA et al., 2009; LEVITZKI; KLEIN, 2010; MARIA et al., 2008). EGFR é codificado por um gene com 237.600 pares de bases (pb) possuindo 28 éxons localizados no cromossomo 7, *locus* 7p11.2. A transcrição do seu DNA produz um mRNA de 5616 pb codificando um precursor de proteína com 1210 aminoácidos (AA) correspondendo 24 AA ao péptido de sinalização e 1186 AA na proteína madura. A proteína EGFR é dividida em três partes: domínio extracelular (ou ectodomínio), um domínio transmembranar e um domínio do tipo tirosina quinase (intracelular). O ectodomínio, por sua vez é subdividido em quatro regiões: os domínios II e IV que são ricos em cisteína e; do domínio I e III, responsáveis pela ligação à

moléculas específicas; o domínio II, braço de dimerização do EGFR diretamente responsável pela dimerização do receptor (Figura 4) (CAPDEVILA et al., 2009; ROSKOSKI, 2014).

Figura 4 – Modelo de dimerização de EGFR.



Fonte: adaptado de WARD e colab., 2007

Após a ligação do fator de crescimento nos domínios I e III ocorrem alterações conformacionais no domínio II, o que leva a sua exposição para dimerização. Além disso, a dimerização do domínio IV próximo à membrana permite aproximar a região C-terminal do ectodomínio dos dois monómeros, permitindo a dimerização do domínio transmembranar (LU et al., 2012). Este processo leva à ativação do domínio tirosina quinase por um mecanismo alostérico no qual dois domínios tirosina quinase formam um dímero assimétrico. O lóbulo C-terminal de um domínio quinase interage com o lóbulo N-terminal do outro e o estabiliza na conformação ativa. Existe uma região de 41 AA entre o domínio transmembranar e o domínio tirosina quinase que permite a estabilização do dímero de quinases assimétrico (LU et al., 2012). A ligação do fator de crescimento ao EGFR leva à sua ativação e consequentemente à ativação de outras vias metabólicas que se localizem *downstream* em relação a ele como as vias metabólicas

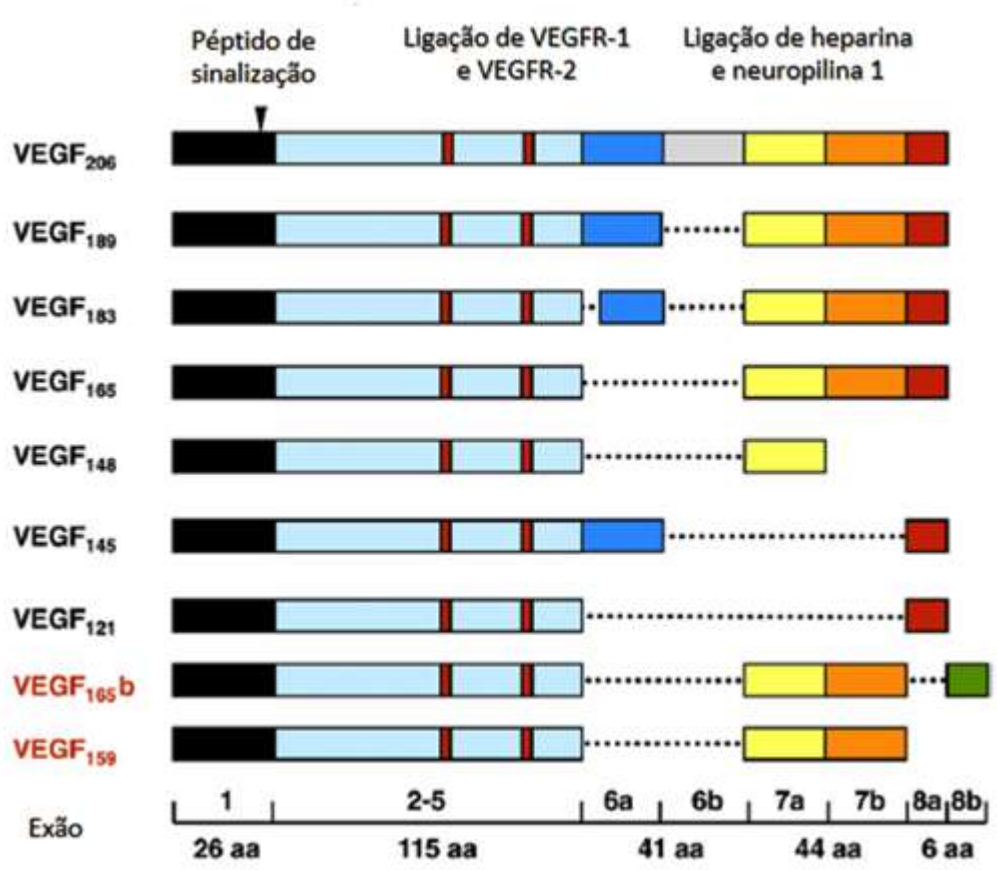
fosfatidilinositol 3-quinase (PI3K)/AKT e RAS-RAF proteína quinase ativada por mitógeno (MAPK). Estas duas vias metabólicas estão envolvidas na migração celular, angiogênese, inibição de apoptose e crescimento celular; todos estes processos estão associados ao desenvolvimento e progressão do câncer (NAKAI; HUNG; YAMAGUCHI, 2016; ROSKOSKI, 2014). Desta forma, a desregulação nesta via metabólica pode levar a carcinogênese, como já foi observado para o aumento da expressão de EGFR em câncer de pulmão, ovário, cervical, mama, coloretal, entre outros (Akhtar et al. 2014; Bellone et al. 2007; Gadducci, Elena, and Greco 2013; Lee et al. 2004).

Devido à sua importância na carcinogênese, a via metabólica do EGFR tornou-se um alvo no desenvolvimento de terapias contra o câncer, podendo ser inibida de duas formas: o uso de anticorpos monoclonais – cetuximab, matuzumab e panitumumab – que se ligam ao ectodomínio de EGFR e atuam através da inibição competitiva com os ligantes de EGFR resultando em internalização do receptor com posterior degradação; ou o uso de pequenas moléculas de natureza química - gefitinib, erlotinib, afatinib e osimertinib – que têm como alvo o domínio de ligação do ATP no domínio tirosina quinase do EGFR como forma a inibir a sinalização da via do EGFR (CAPDEVILA et al., 2009; NAKAI; HUNG; YAMAGUCHI, 2016).

2.3.2 Fator de Crescimento Vascular Endotelial (VEGF) e receptores

A família do fator de crescimento vascular endotelial (VEGF) é constituída por fator mitogênico e de sobrevivência para células endoteliais, mobilidade de monócitos, vasodilatação e aumento de permeabilidade vascular, fatores necessários para a ocorrência de angiogênese (NAGY; DVORAK; DVORAK, 2007; ROSKOSKI, 2007). São cinco as moléculas desta família: VEGFA (VEGF), fator de crescimento placentário (PIGF), VEGFB, VEGFC e VEGFD (ROSKOSKI, 2008). O gene que codifica para VEGF é constituído por 8 éxons e 7 íntrons, mas o *splicing* alternativo leva ao desenvolvimento de diversas isoformas de VEGF (Figura 5) (FERRARA et al., 2004) constituídas por 121, 145, 148, 165, 189 ou 206 AA (HOEBEN et al., 2004).

Figura 5 - Estrutura das isoformas de VEGF.



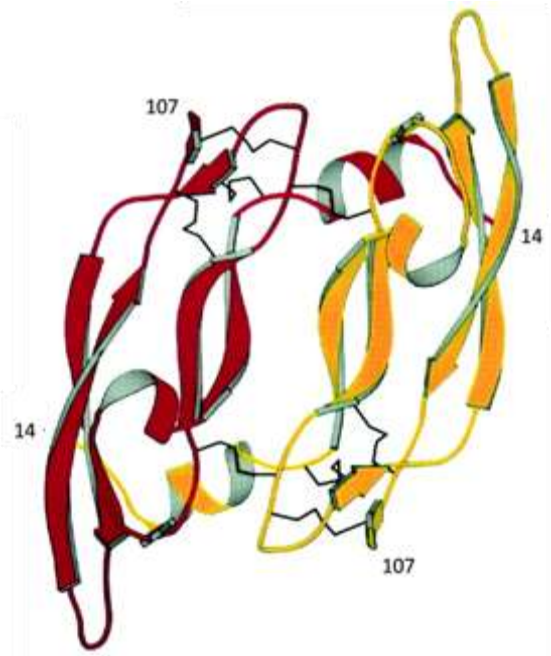
Fonte: adaptado de GRÜNEWALD e colab., 2010.

VEGF tem a estrutura de uma glicoproteína com pontes dissulfeto com tamanho entre 34 e 42kDa e apresenta-se na forma dimérica (HOEBEN et al., 2004). VEGF é encontrado superexpresso em vários tipos de câncer, incluindo de mama, coloretal e próstata (ROSKOSKI, 2007). As isoformas de VEGF podem ser do tipo $VEGF_{XXX}$ ou $VEGF_{XXX}b$ desde que haja integração do éxon 8a ou 8b, respectivamente. As VEGF-165a e -121a são as isoformas pro-angiogênicas mais abundantes, enquanto que as VEGF-165b e -121b são as anti-angiogênicas mais reportadas (DELCOMBEL et al., 2013).

O domínio de ligação do VEGF é constituído pelos aminoácidos 14 até 107 (Figura 6). Estudos da estrutura cristalográfica do VEGF entre os resíduos 8 e 109 revelou que esta proteína forma um homodímero antiparalelo conectado através de ponte dissulfeto entre os AA Cys51 e Cys60. Além disso, a principal característica de

cada homodímero é um anel de 8 resíduos formados pelas pontes dissulfeto Cys57-Cys102 e Cys61-Cys102 com uma outra ponte dissulfeto entre as Cys26-Cys68, formando desta forma um nó (ROSKOSKI, 2007).

Figura 6 – Homodímero antiparalelo de VEGF.



Fonte: adaptado de SHINKARUK e colab., 2003.

O VEGF desempenha papel fundamental na neovascularização tumoral. Durante o desenvolvimento do tumor, células dentro da massa tumoral em expansão frequentemente sofrem privação de oxigênio (hipóxia) devido à distância do vaso sanguíneo mais próximo, assim começam a formar-se regiões de hipóxia. Este fenômeno leva à produção de VEGF por dois mecanismos independentes: aumento da expressão da VEGF e; estabilização do mRNA de VEGF. Adicionalmente, a inativação de p53 e expressão do oncogene Src parecem levar a um aumento da expressão de VEGF.

São diversos os reguladores da angiogênese tumoral, como citocinas, hormônios, fatores de crescimento moduladores de VEGF em diferentes ambientes celulares, e assim exibem efeito angiogênico indireto. A permeabilidade vascular, migração de células endoteliais para os alvos que serão vascularizados, e para a formação de uma nova malha de capilares ativos são causados pela ativação da via do EGFR e produção de metaloproteases decorrentes do aumento de expressão de VEGF junto com a secreção de fatores de crescimento. Portanto, a via do VEGF tem um papel

predominante na angiogênese tumoral e desenvolvimento do câncer (SHINKARUK et al., 2003).

O VEGF pode-se ligar a três diferentes proteínas receptoras de tirosina quinase: VEGFR1 (Flt1), VEGFR2 (KDR) e VEGFR3 e dois receptores não enzimáticos (neuropilina-1 e -2). Cada um destes receptores é do tipo tirosina quinase V (cinco), consistindo num componente extracelular contendo sete domínios *immunoglobulin-like*, um segmento transmembrana e um domínio tirosina quinase que contém de 70 a 100 resíduos de aminoácidos, e uma cauda carboxila terminal (ROSKOSKI, 2008).

Diversas isoformas da família VEGF tem afinidade com proteoglicanos heparano-sulfato encontrados na membrana celular e matriz extracelular, enquanto que os receptores de VEGF apresentam diferentes afinidades e eficiências de sinalização. O VEGFR1 apresenta maior afinidade com VEGF e possui uma menor atividade tirosina quinase que o VEGFR2. Apesar disso, o VEGFR2 apresenta uma atividade tirosina quinase forte comparado com o VEGFR1. Em células vasculares endoteliais VEGF, liga-se a VEGFR1 e VEGFR2 (ROSKOSKI, 2008), levando à dimerização, a ativação da atividade tirosina quinase e consequente ao desencadeamento da cascata de sinalização. (ROSKOSKI, 2008).

A inibição da interação entre o VEGF e seus receptores leva à repressão da angiogênese e crescimento tumorais. Além disso, pode levar também à prevenção de metástases pelo reduzido contato entre a massa tumoral e os vasos sanguíneos (ROSKOSKI, 2007; SHINKARUK et al., 2003). Desta forma, o VEGF tornou-se um importante alvo terapêutico em câncer, assim como em doenças oftalmológicas (NAGY; DVORAK; DVORAK, 2007) Em pacientes com câncer do cólon avançado, o uso de bevacizumab permite retardar a recorrência da neoplasia e aumenta a esperança de vida em cerca de 4 a 5 meses (NAGY; DVORAK; DVORAK, 2007).

A terapia direcionada para o VEGF levou a melhores resultados em vários tumores sólidos. Vários estudos mostraram a correlação entre expressão de VEGF, mau prognóstico e recorrência precoce de câncer cervical. Além disso, níveis baixos de VEGF estão associados com melhor resposta tumoral à ao tratamento simultâneo com quimioterapia e radioterapia (ROSKOSKI, 2008).

2.4 BIOINFORMÁTICA

De acordo com o National Institute of Health, bioinformática é definida como a pesquisa, desenvolvimento ou aplicação de ferramentas computacionais e abordagens para expansão do uso de dados biológicos, médicos, comportamentais ou de saúde incluindo adquirir, armazenar, organizar, arquivar, analisar ou visualizar esses dados. Trata-se de uma ciência interdisciplinar incluindo conhecimentos de física, bioquímica e informática (CHEN; KURGAN, 2012). Com a chegada de tecnologias de alto rendimento e avanços no campo da bioinformática e biologia computacional pesquisadores são capazes de gerar, acessar analisar e interpretar diversos conjuntos de dados e integrá-los numa escala que não era possível (KORCSMAROS; SCHNEIDER; DE, 2017). Este campo é de ampla aplicação em pesquisa como análise de sequências, anotações genômicas, biologia da evolução e mesmo para prever consequências funcionais e estruturais de mutações (CHEN; KURGAN, 2012), que podem afetar a forma como elas funcionam direcionando as possíveis terapias em doenças como o câncer (BORBA et al., 2016). Para determinar as consequências decorrentes de mutação em proteínas foram desenvolvidas diversas ferramentas informáticas, algoritmos, que tomam como argumentos as propriedades das proteínas, e permitem obter uma avaliação do seu potencial deletério, como SIFT, Polyphen, entre outros (LIU et al., 2016).

Os algoritmos para avaliação de impacto de mutações, entre outros são baseados em diferentes técnicas de *machine learning* (aprendizagem de máquinas) como *Support Vector Machine* (SVM), *Random Forest* (RF), *Naive Bayes* ou regressão. O mais clássico e consequentemente mais utilizado é o SVM, que foi desenvolvido numa base de aprendizagem estatística teórica para maximizar as margens de separação entre exemplos de duas classes projetadas em um hiperespaço (LI et al., 2009). Trata-se de um método de reconhecimento de padrões do tipo *kernel-based* que utiliza a aprendizagem de máquinas. A diferença do SVM para os demais métodos de classificação é que os modelos de SVM dependem apenas das amostras próximas dos limites entre duas (ou mais) classes – ou seja, as amostras nas margens de cada classe, que são chamadas de vetores de suporte (*support vectors*) (LIU et al., 2013).

O RF é um conjunto de árvores em um espaço vetorial de múltiplas dimensões de variáveis de um objeto classificado, seu resultado é baseado em uma média dos

resultados independentes de cada árvore (LI et al., 2009; LIU et al., 2013). O método de *Neural Network,* ou Redes Neurais, mimetiza o funcionamento do cérebro humano e tem sido cada vez difundido por dois motivos principais: (i) como as propriedades das sequências proteicas estão distribuídas em hiperespaços com características complexas, normalmente é difícil encontrar modelos satisfatórios usando abordagens estatísticas ou parametrizadas; (ii) métodos baseados em NN são capazes de processar valores contínuos apresentados ao modelo (CAO; XIONG, 2014).

Apesar de utilizarem métodos diversos para classificar os polimorfismos, os algoritmos partem de dados biológicos similares para realizar as análises. Alguns baseiam suas análises exclusivamente em dados de conservação obtidos através de alinhamento de múltiplas sequencias, como o Mutation Assessor e o SIFT. Outras abordagens, como as aplicadas pelo PolyPhen2, SNPs&GO e MutPred, combinam informações de homologia com vários tipos de anotações funcionais e estruturais, tais como: (i) propriedades físico-químicas dos aminoácidos envolvidos na troca; (ii) localização de regiões funcionais; (iii) estrutura secundária e (iv) topologia da proteína (FROUSIOS et al., 2013).

Desta forma, o uso de ferramentas computacionais tem contribuído para análise do impacto de mutações em várias situações seja em avaliação de atividade de enzimas metabolizadoras de medicamentos como a P450 (codificada pelo gene CYP2D6) (BORBA et al., 2016) e a CYP2C19 (DING et al., 2015), assim como em estudos da sensibilidade a gefitinib em pacientes com mutações no EGFR (RAGHAV; SHARMA; AGARWAL, 2013). Além disso, estas ferramentas permitiram identificar mutações no gene da adiponectina potencialmente associadas com diabetes, obesidade e inflamação (A; VALASALA; KAMMA, 2015) assim como mutações no exoma que podem ter papel na síndrome de morte súbita (SUKTITIPAT et al., 2017).

3 OBJETIVOS

3.1 OBJETIVO GERAL:

Identificar biomarcadores específicos para auxiliar no diagnóstico, prognóstico e direcionamento terapêutico em câncer cervical.

3.2 OBJETIVOS ESPECÍFICOS:

- Identificar genes que podem estar relacionados à regulação celular, desde a infecção por HPV até o câncer cervical;
- Identificar genes relacionados ao desenvolvimento do câncer cervical: ao nível de diagnóstico e de prognóstico utilizando ferramentas computacionais;
- Identificar possíveis marcadores moleculares para alvos terapêuticos baseados em anticorpos;
- Correlacionar os dados laboratoriais com os dados clínicos de pacientes submetidos à tratamento oncológico.

4 MANUSCRIPT 1:

EXPRESSION PROFILES OF IL-10, SIRT1, SIRT3, AND SIRT5 IN CERVICAL CANCER DEVELOPMENT

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EXPRESSION PROFILES OF IL-10, SIRT1, SIRT3, AND SIRT5 IN CERVICAL CANCER DEVELOPMENT

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Abstract

Background: Cervical cancer is the fourth most common cancer among women, caused by HPV infection. The aim of this study was to identify the differential expression of IL-10, SIRT1, SIRT3, and SIRT5 molecules in HPV infected samples and cancer samples.

Methods: A total of 217 samples were evaluated for HPV infection, and 114 samples were analyzed for gene expression levels of IL-10, SIRT1, SIRT3 and SIRT5.

Results: 96.9% of cervical smear samples were valid 12.69% infected with HPV; while 100% of the cancer samples were valid and HPV-infected. IL-10 was upregulated in cervical smears and downregulated in cervical cancer samples (p=0.0005). SIRT1 showed a discrete downregulation in both HPV^+ and cervical cancer samples, while SIRT5 was the only gene overexpressed in cervical cancer, compared to IL-10 and SIRT1 ($p<0.0001$ and $p=0.0014$, respectively). SIRT3 was only detected in cancer group with significant lowers levels than SIRT1 and SIRT5 ($p=0.008$ and $p=0.015$, respectively),

Conclusions: Although the extension of SIRT5 overexpression in cervical cancer are not clear, it is involved in reduction of reactive oxygen species (ROS), suggesting an antagonist of HPV-induced hypoxia. However, further studies are necessary to elucidate these mechanisms further, as the SIRTs functions appear be related to specific tissues.

Keywords: HPV, Cervical cancer; Sirtuins, Interleukin-10.

Background

Human papillomavirus (HPV) is the primary etiological factor for the development of cervical cancer [1], the fourth most frequent cancer in women worldwide, which near to 80% occurring in developing countries [2].

Epithelial cells infected by HPV are able to block interferon type I (IFN-I) pathways as part of an immune evasion mechanism. As lesions progress to invasive carcinoma, there is an increase in the number of infiltrating macrophages M2-like phenotype expressing TGF-β and IL-10. It contributes to the stimulation of Treg cells, suppressing the antitumor activity of CD8 CTL lymphocytes [3]. However, IL-10 was also associated with poor prognosis in a variety of cancers (melanoma, lung and T/NK-cell lymphomas) due to the decrease in anti-tumor immune response [4]. In cervical cancer, IL-10 was found highly expressed in keratocytes and macrophages, being associated with viral persistence infection and cervical carcinogenesis progression [5].

By another side, HPV infected cells lead to the deregulation of the cell cycle through inhibition/degradation of different proteins like, pRB and p53. These oncogenes are responsible for arresting the cell in G1 phase, and found deregulated in many cancers [6]. In this context, sirtuins (SIRTs) have demonstrated to be important in normal and diseased cells, including cancer cells that exhibit adapted metabolism to support cell growth and division. SIRTs are able to inhibit these processes, and regulate pathways of differentiation, cell adhesion, cell–cell communication and inflammation, opposing the cancer- associated metabolic pathway alterations and uncontrolled proliferation [7,8]. Sirtuin family has 7 class-III histone deacetylases that catalyze NAD^+-dependent deacetylation or ribosylation of histones and non-histones proteins, controlling the interactions of histones with DNA, the chromatin conformation and transcriptional activation [9].

In BCR–ABL- transformed cells, the inhibition or genetic loss of SIRT1 leads to p53 activation, suppressed growth and apoptosis [8,10], while upregulation of SIRT1 in breast cancer was associated with distant metastasis and poor prognosis [11]. SIRT3 deregulated expression was found associated with cell proliferation in gastric cancer, prevention of apoptosis in glioma cells and resistance to insulin in mice [12], and SIRT5 is considered a oncogene in non-small-cell lung cancer [7]. The aim of this study was to determine the expression patterns of IL-10, SIRT1, SIRT3 and SIRT5 in cervical smear of patients infected with HPV and tissue samples from cervical cancer.

Methods

Samples

Cervical samples were collected from 195 women in a Primary Care Unit in Pesqueira, Pernambuco, Brazil, following a transversal cohort design. Two cytobrushes were collected and maintained in phosphate-buffered saline (PBS) pH 7.0 (Life Technologies, USA), or TRIzol® reagent, at -20ºC until molecular analysis. A total of 22 cervical cancer tissues were collected from patients during cervical surgery in the Clinical Hospital, Pernambuco, Brazil, and maintained in TRIzol® reagent at -20ºC until molecular analysis. All patients signed the inform consent approved by institutional ethical committee Health Sciences Center from Federal University of Pernambuco, Brazil (Codes No. 852.334 and 522.239) and all research was conducted according to the guidelines of the Declaration of Helsinki (1964).

Nucleic acid purification

Cytological brushes samples stored in PBS or in TRIzol® were thawed, mixed by vortex for 10 sec prior to removal of the brush. Samples were centrifuged for 2 min at

14,000 rpm and pellet was used for nucleic acid purification. DNA purification was performed using Wizard Genomic DNA purification kit (Promega, USA) following manufacturer's instructions. Eluted DNA was stored at -20ºC until further processing. RNA purification was performed with DirectZol™ RNA Miniprep (Zymo Research, USA), following manufacturer's instructions. Eluted RNA was quantified in NanoDrop® – 2000 Spectrophotometer (Thermo Fisher Scientific, USA) and maintained at -80oC until molecular processing.

Fresh cervical tissues used were thawed and cut in 30mg pieces to perform DNA and RNA purification through DNaeasy Blood and Tissue kit (Qiagen, USA) and TRIzol® reagent (Thermo Fisher Scientific, USA), respectively, following the manufacturer's instructions. RNA concentration was determined and stored at -80ºC until further processing.

HPV infection

DNA purified from cervical cells and cancer tissues was used to determine the presence of HPV infection through PCR amplification. HPV infection was evaluated using consensus primers MY09/11 [13] and $GP5^{+}/6^{+}$ [14], while the quality of the DNA was determined by β-globin amplification as reference gene [15]. PCR reaction was prepared for 12.5µl final volume, as follows: 6.25uL of Promega Master Mix Green (Promega, USA), 1uL forward primer (10pmol/µl), 1uL reverse primer (10pmol/µl), 1uL of eluted DNA and 3.25uL ultrapure water. The PCR setup was initial denaturation at 95ºC for 2 min; followed by for 35 cycles of 95ºC for 1 minute, 55ºC for 1 min for MY09/11 or and 45ºC for $GP5^{+}/6^{+}$, 72ºC for 1 min; and final extension at 72ºC for 5 minutes. All PCRs were performed in Veriti Thermal Cycler (Applied Biosystems, USA) and amplification results were observed in 1% agarose gel prepared in SB buffer

(boric acid 363.9μM and NaOH 1mM). Electrophoresis was carried at 120V and observed under UV light in Epi-light (Loccus Biotecnologia, Brazil) using 0.5μg/ml of ethidium bromide. Samples were considered valid for further analysis after β-globin amplification. HPV-positive results were assumed for samples with at least two positive results in HPV amplification.

Gene expression

Complementary DNA (cDNA) was obtained using QuantiTec Reverse Transcription Kit® (Qiagen, USA) for RNA purified from cervical cells, and QuantiNova Reverse Transcription Kit® (Qiagen, USA) for RNA purified from cervical cancer samples, following manufacturer's instructions. The qPCR experiments were carried in RotorGene Q (Qiagen, USA) equipment with RotorGene SybrGreen MasterMix (Qiagen, USA) and QuantiNova Sybr Green Mastermix (Qiagen, USA) for samples from cervical cells and cervical cancer tissue, respectively. RPLP0 was used as housekeeping gene [16] for the specific primers obtained from IDT (Integrated DNA Technology, USA) as commercial assays: IL-10 (Hs.PT.58.40790669), SIRT1 (Hs.PT.58.2807216), SIRT3 (Hs.PT.58.22633153.g), SIRT5 (Hs.PT.58.38597405). All samples were measured twice with NTC (no template control) included in all runs, within detection limit of Ct 38 assumed as valid after evaluation of melting curve.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 7.0 (GraphPad Software, CA). Pearson correlation test and the t-Student test were used to investigate the hypothesis significance. It was considered statistically significant $p<0.05$.

Results

Of the 195 cervical smears evaluated, 96.92% (189/195) were valid and 12.69% (24/189) showed HPV^+ results in PCR analysis. All 22 cervical cancer samples were considered valid for molecular analysis and found infected by HPV. Therefore, samples were divided in 3 groups for mRNA expression analysis: 24 samples in HPV^+ group; 68 samples in HPV^- group; and 22 samples in Cancer group. The mean age of the groups present in this study was 41.0±13.1 years in the HPV^- group, 41.6±11.5 in HPV^+ group and 43.0±11.8 in the cervical cancer group.

Analysis of IL-10 mRNA showed upregulation in HPV^+ group compared to HPV^- group, but a significant decrease can be observed in cancer samples (p=0.0005) (Figure 1). In HPV^+ group, IL-10 expression showed a similar profile of SIRT5, both upregulated (Figure 2), while SIRT1 was downregulated in this group.

In cancer, IL-10 and SIRT1 were downregulated, but showed statistical significance (p=0.0034). SIRT5 was the only upregulated molecule in cancer samples, showing statistical significance for IL-10 (p<0.0001) and SIRT1 (p=0.0014) (Figure 3).

SIRT3 did not show expression in the HPV groups, being only detected in cancer samples. Evaluation of ΔCt showed significant lower levels compared to SIRT1 and SIRT5 (p=0.008 and p=0.015, respectively) (Figure 4).

No difference was observed for smoking or drinking habits, parity and cancer stage for IL-10, SIRT1, SIRT3 and SIRT5 in cancer group.

Discussion

IL-10 production is modulated by high-risk HPV and it has been found overexpressed in high-grade cervical lesions and cervical cancer samples, allowing the HPV infection persistence and neoplastic progression [5]. The progression from LSIL

(low-grade squamous intraepithelial lesion) and HSIL (high-grade squamous intraepithelial lesion) to cancer shows a significant increase in IL-10 expression [17], [18] suggesting that the overexpression increases according to the lesion severity to cervical cancer [19]. Unlikely, our study showed IL-10 overexpressed in HPV^+ samples but downregulated in cervical cancer samples. Since IL-10 may have tumor-promoting or -inhibiting effects at different concentrations and in different environments, some researchers have hypothesized that higher levels of IL-10 promote HPV growth, viral replication, and malignant transformation of infected cells in women infected with the virus. This is a possible explanation of why some women with HPV get cervical cancer while others do not [20].

In cervical cancer samples, SIRT1 overexpression was already reported as good biomarker for disease progression [23], found highly expressed in pre-neoplastic lesions compared to the non-neoplastic group [24]. In SiHa cell lines ($HPV16^+$), the silencing of SIRT1 leads to apoptosis [25]. Curiously, SIRT1 relative expression was found slightly downregulated in HPV^+ group without cervical lesion and also in cancer group, but both with a heterogeneous profile. This behavior was already observed in cervical squamous cell carcinoma [24].

In HPV infected cells, SIRT3 functions as a tumor suppressor by limiting ROS, antagonizing hypoxia-inducible factor 1 (HIF1) that is induced by HPV in the microenvironment [27]. It was also found protecting HeLa cells ($HPV18^+$) from genotoxic and oxidative stress-mediated cell death, while it was downregulated in breast cancer [28]. SIRT3 functions are controversial once it is related to cell death and survival. Our data demonstrate the absence of SIRT3 expression in HPV groups but detectable levels in cancer cells, which could be related to the constitutive expression of HPV E6 and E7 integrated to the human genome. This would maintain the levels of E6

that induces an increase in HIF-1α under hypoxic conditions through degradation of p53, a tumor suppressor protein that binds to and induces HIF-1α degradation [29]. Thus, leads to cell proliferation through the metabolic shift from oxidative phosphorylation to glycolysis and lactic acid production, and stimulation of epithelial-mesenchymal transition, angiogenesis, autophagy, and synthesis and storage of lipid and glycogen for providing nutrient supply [30]. This condition could be enhanced by SIRT5 that supports the use of alternative energy sources under conditions of limited nutrient availability [31].

SIRT5 was found overexpressed in pancreas adenocarcinoma [32] and in non-small-cell lung cancer (NSCLC) [33], as observed in our cancer group. However, in other types of cancer, as head and neck squamous cell carcinoma (HNSCC) SIRT5 was found downregulated [34,35], indicating no consensus about the role of SIRT5 in neoplasia. In the mitochondria, SIRT5 binds to and desuccinylates the superoxide dismutase (SOD1), an enzyme responsible for the removal of the reactive oxygen species (ROS) generated during the normal mitochondrial functioning [36]. Oncogenic transformation activates proliferative reprogramming pathways that generate ROS [37]; a molecule necessary for hypoxic activation of HIF [38]. In cervical cancer, the hypoxic microenvironment can be maintained due to the continuous expression of HPV E6, and consequent HIF1 stimulation [29], once promotes the storage of glucose in the form of glycogen in nonmalignant as well as in cancer cells for later use under nutrient-limiting conditions [39]. Cancer cells are also likely to use autophagy to obtain amino acids as alternative energy sources [30]. So, high levels of SIRT5 seems to have an tumor–inhibiting effect, once the presence or overexpression of SIRT5 could inhibit the protective effects of autophagy toward stressful conditions such as for example hypoxia or chemotherapy

[40], decreasing ROS levels and inhibiting HIF1, though SIRT5 upregulation was found not only facilitating lung cancer growth but also associated with drug resistance [33].

Conclusions

The roles of cytokines and sirtuins in HPV infection and cervical cancer development are not fully understood. SIRT3 and SIRT5 expression in cervical cancer could be a response mechanism to regulate the HIF-1α induced by E6 ubiquitination of p53. However, the role of sirtuins seems to be tissue specific, so further studies should be pursued to elucidate these mechanisms in cervical cancer.

List of abbreviations

IL-10 – interleukin 10; INF-I – interferon type; HIF – hypoxia inducible factor; HPV – human papillomavirus; HSIL – high-grade squamous intraepithelial lesion; LSIL - Low-grade squamous intraepithelial lesion; NSCLC – non-small cell lung cancer; NTC – no template control; ROS – reactive oxygen species; RPLP0 – ribosomal protein, large, P0; SIRT – sirtuin; SOD1 – superoxide dismutase 1; TGF-β – tumor growth factor β;

Ethics approval and consent to participate

All patients signed the inform consent approved by institutional ethical committee Health Sciences Center from Federal University of Pernambuco, Brazil (Codes No. 852.334 and 522.239).

Competing interests

The authors declare no conflict of interests.

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Author's contributions

MFSC contributed in the molecular analysis and manuscript preparation; ACSA, PRA and IMG were responsible for collecting the biological samples, patient's record and inform consent; SEBS and MHFE contributed in processing the samples for molecular analysis; JLLF and JACT were scientific collaborator for this project and DBGM designed the study, evaluated the results and the final manuscript. All the authors read and approved the final manuscript.

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References

1. Lees BF, Erickson BK, Huh WK. Cervical cancer screening: Evidence behind the guidelines. Am. J. Obstet. Gynecol. [Internet]. Elsevier Inc.; 2016;214:438–43. Available from: http://dx.doi.org/10.1016/j.ajog.2015.10.147

2. Ferlay J, Soerjomataram I, Ervik M, Dikshit R, Eser S, Mathers C, et al. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [Internet]. Lyon, Fr. Int. Agency Res. Cancer; 2013. 2012 [cited 2016 Oct 28]. Available from: http://globocan.iarc.fr

3. Boccardo E, Lepique AP, Villa LL. The role of inflammation in HPV carcinogenesis. Carcinogenesis. 2010;31:1905–12.

4. Mannino MH, Zhu Z, Xiao H, Bai Q, Wakefield MR, Fang Y. The paradoxical role of IL-10 in immunity and cancer. Cancer Lett. 2015;367:103–7.

5. Prata TTM, Bonin CM, Ferreira AMT, Padovani CTJ, Fernandes CE dos S, Machado AP, et al. Local immunosuppression induced by high viral load of human papillomavirus: Characterization of cellular phenotypes producing interleukin-10 in cervical neoplastic lesions. Immunology. 2015;146:113–21.

6. Cardoso M de FS, Castelletti CHM, Lima-Filho JL de, Martins DBG, Teixeira JAC. Putative biomarkers for cervical cancer: SNVs, methylation and expression profiles. Mutat. Res. Mutat. Res. Elsevier B.V.; 2017;773:161–73.

7. Chalkiadaki A, Guarente L. The multifaceted functions of sirtuins in cancer. Nat. Rev. Cancer. 2015;15:608–24.

8. Q W, C Y, M X, L H, Y Z, M S. Sirtuin 1 (Sirt1) Overexpression in BaF3 Cells Contributes to Cell Proliferation Promotion, Apoptosis Resistance and Pro-Inflammatory Cytokine Production. Med. Sci. Monit. 2017;23:1477–82.

9. Witt O, Deubzer HE, Milde T, Oehme I. HDAC family: What are the cancer relevant targets? Cancer Lett. 2009;277:8–21.

10. Li L, Wang L, Li L, Wang Z, Ho Y, McDonald T, et al. Activation of p53 by SIRT1 inhibition enhances elimination of CML leukemia stem cells in combination with imatinib. Cancer Cell. 2012;21:266–81.

11. Yuan H, Su L, Chen W. The emerging and diverse roles of sirtuins in cancer: A clinical perspective. Onco. Targets. Ther. 2013;6:1399–416.

12. Parihar P, Solanki I, Mansuri ML, Parihar MS. Mitochondrial sirtuins: Emerging roles in metabolic regulations, energy homeostasis and diseases. EXG. 2015;61:130–41.

13. Manos MM, Ting DK, Wright AJ, Lewis TR, Broker TR, Wolinsky SM. The use of polymerase chain reaction amplification for the detection of genital human papillomaviruses. Cancer Cells. 1989;7:209–14.

14. Snijders PJF, Brule D, Husman DR, Husman DR. The use of general primers GP5 and GP6 elongated at their 3 â€^{TM} ends with adjacent highly conserved sequences improves human papillomavirus detection by PCR. J. Gen. Virol. 1995;76:1057–62.

15. Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, et al. Enzymatic Amplification of , -Globin Genomic Sequences and Restriction Site Analysis for Diagnosis of Sickle Cell Anemia. Science (80-.). 230:1350–2.

16. Tan SC, Ismail MP, Duski DR, Othman NH, Bhavaraju VMK, Ankathil R. Identification of Optimal Reference Genes for Normalization of RT-qPCR Data in Cancerous and Non-Cancerous Tissues of Human Uterine Cervix. Cancer Invest. 2017;1–11.

17. Peghini BC, Abdalla DR, Barcelos ACM, Teodoro L das GVL, Murta EFC, Michelin MA. Local cytokine profiles of patients with cervical intraepithelial and

invasive neoplasia. Hum. Immunol. 2012;73:920–6.

18. Azar KK, Tani M, Yasuda H, Sakai A, Inoue M, Sasagawa T. Increased secretion patterns of interleukin-10 and tumor necrosis factor-alpha in cervical squamous intraepithelial lesions. Hum. Pathol. 2004;35:1376–84.

19. Ali KS, Ali HYM, Jubrael JMS. Concentration levels of IL-10 and TNF α cytokines in patients with human papilloma virus (HPV) DNA + and DNA − cervical lesions. J. Immunotoxicol. 2012;9:168–72.

20. Langsenlehner U, Oppenheim J, Sciences B. Researchers Attempting To Define Role of Cytokines in Cancer Risk. J. Natl. Cancer Inst. 2005;97:1175–7.

21. Shibata M, Nezu T, Kanou H, Nagata Y, Kimura T, Takekawa M, et al. Immunomodulatory effects of low dose cis-Diaminedichloroplatinum (cisplatin) combined with UFT and PSK in patients with advanced colorectal cancer. Cancer Invest. 2002;20:166–73.

22. Tadagavadi RK, Reeves WB. Endogenous IL-10 attenuates cisplatin nephrotoxicity: role of dendritic cells. J. Immunol. 2010;185:4904–11.

23. Velez-Perez A, Wang XI, Li M, Zhang S. SIRT1 overexpression in cervical squamous intraepithelial lesions and invasive squamous cell carcinoma. Hum. Pathol. 2016;59:102–7.

24. Singh S, Kumar PU, Thakur S, Kiran S, Sen B, Sharma S, et al. Expression/localization patterns of sirtuins (SIRT1, SIRT2, and SIRT7) during progression of cervical cancer and effects of sirtuin inhibitors on growth of cervical cancer cells. Tumor Biol. 2015;36:6159–71.

25. Ford J, Jiang M, Milner J. Cancer-specific functions of SIRT1 enable human epithelial cancer cell growth and survival. Cancer Res. 2005;65:10457–63.

26. Cao B, Shi Q, Wang W. Higher expression of SIRT1 induced resistance of esophageal squamous cell carcinoma cells to cisplatin. J. Thorac. Dis. 2015;7:711–9.

27. George J, Ahmad N. Mitochondrial sirtuins in cancer: Emerging roles and therapeutic potential. Cancer Res. 2016;76:2500–6.

28. Alhazzazi TY, Kamarajan P, Verdin E, Kapila YL. SIRT3 and cancer: Tumor promoter or suppressor? Biochim. Biophys. Acta - Rev. Cancer. Elsevier B.V.; 2011;1816:80–8.

29. Cuninghame S, Jackson R, Zehbe I. Hypoxia-inducible factor 1 and its role in viral carcinogenesis. Virology. Elsevier; 2014;456–457:370–83.

30. Brahimi-Horn MC, Bellot G, Pouysségur J. Hypoxia and energetic metabolism. Curr. Opin. Genet. Dev. 2011;21:67–72.

31. Gertz M, Steegborn C. Function and regulation of the mitochondrial Sirtuin isoform Sirt5 in Mammalia. Biochim. Biophys. Acta - Proteins Proteomics. Elsevier B.V.; 2010;1804:1658–65.

32. Ouaısi M, Sielezneff I, Silvestre R, Sastre B, Bernard J-P, Simony Lafontaine J, et al. High Histone Deacetylase 7 (HDAC7) Expression Is Significantly Associated with Adenocarcinomas of the Pancreas. Ann. Surg. Oncol. 2008;15:2318–28.

33. Lu W, Zuo Y, Feng Y, Zhang M. SIRT5 facilitates cancer cell growth and drug resistance in non-small cell lung cancer. Tumor Biol. 2014;35:10699–705.

34. Yang L, Ma X, He Y, Yuan C, Chen Q, Li G, et al. Sirtuin 5: a review of structure, known inhibitors and clues for developing new inhibitors. Sci. China Life Sci. 2016;60:1–8.

35. Lai C-C, Lin P-M, Lin S-F, Hsu C-H, Lin H-C, Hu M-L, et al. Altered expression of SIRT gene family in head and neck squamous cell carcinoma. Tumor Biol.

2013;34:1847–54.

36. Kumar S, Lombard DB. Mitochondrial sirtuins and their relationships with metabolic disease and cancer. Antioxid. Redox Signal. 2015;22:1060–77.

37. Schumacker P. Reactive Oxygen Species in Cancer: A Dance with the Devil. Cancer Cell [Internet]. Elsevier Inc.; 2015;27:156–7. Available from: http://dx.doi.org/10.1016/j.ccell.2015.01.007

38. Bell EL, Klimova TA, Eisenbart J, Schumacker PT, Chandel NS. Mitochondrial reactive oxygen species trigger hypoxia-inducible factor-dependent extension of the replicative life span during hypoxia. Mol. Cell. Biol. 2007;27:5737–45.

39. Ros S, Schulze A. Linking glycogen and senescence in cancer cells. Cell Metab. Elsevier Inc.; 2012;16:687–8.

40. Polletta L, Vernucci E, Carnevale I, Arcangeli T, Rotili D, Steegborn C, et al. SIRT5 regulation of ammonia-induced autophagy and mitophagy. Autophagy. 2015;11:253–70.

Figure 1 – Expression pattern of IL-10 in cervix scrape and cervical cancer samples.

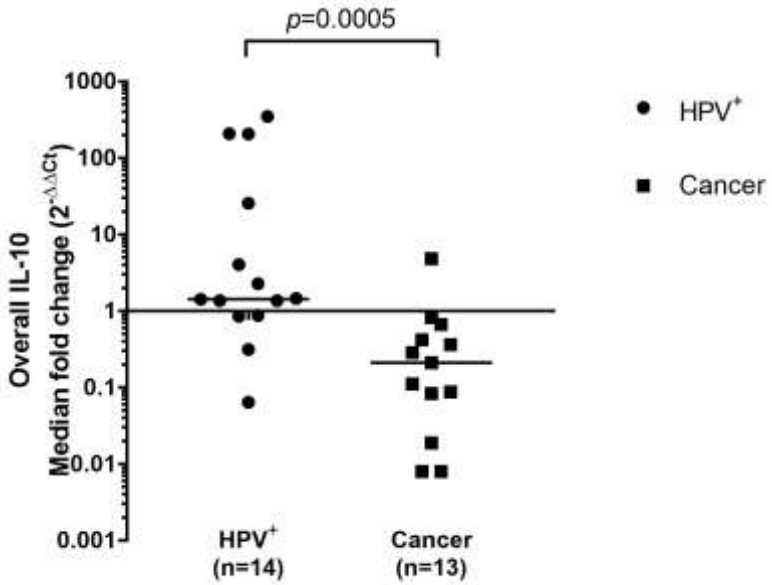


Figure 2 – Fold change in expression of IL-10, SIRT1 and SIRT5 in HPV^+ samples.

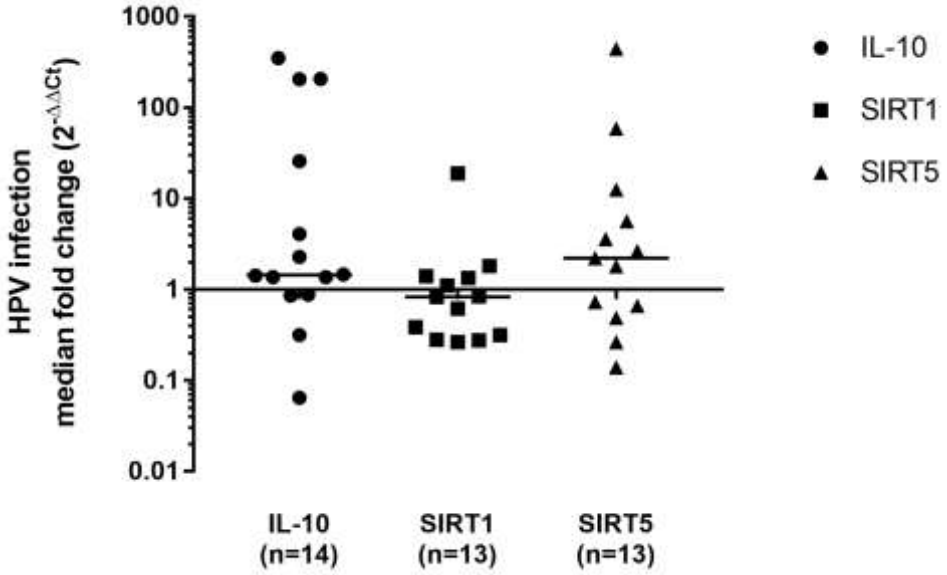


Figure 3 – Fold change in expression of IL-10, SIRT1 and SIRT5 in cervical cancer samples.

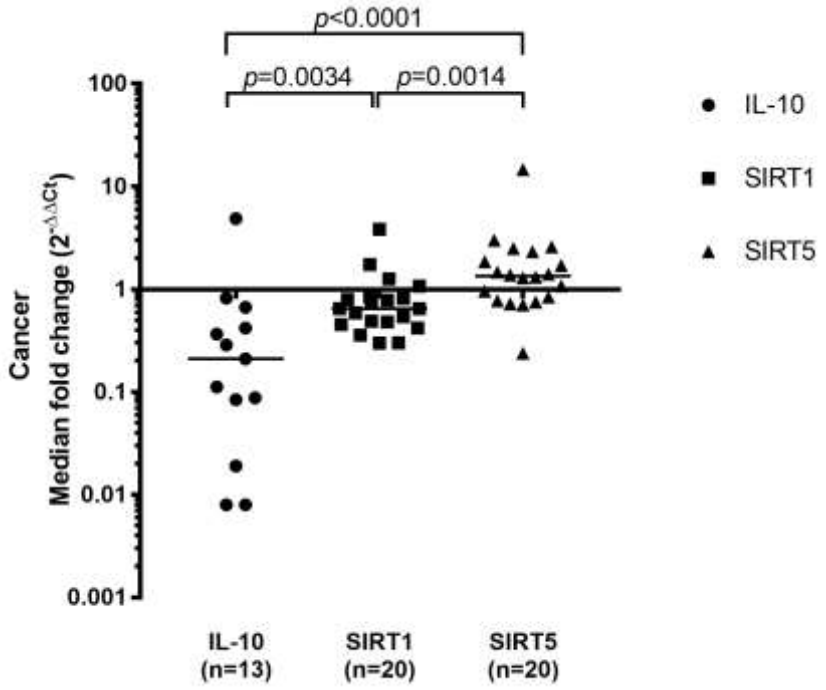
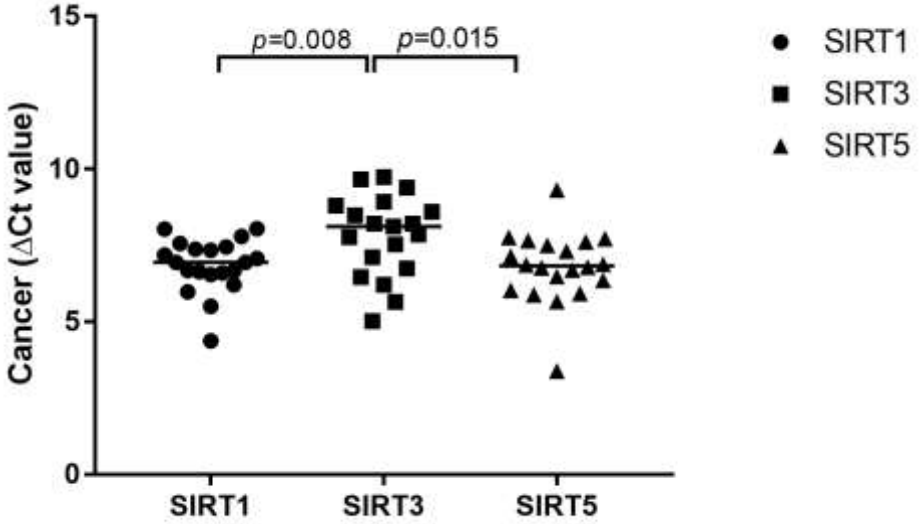


Figure 4 – Relative expression of SIRT1, SIRT3 and SIRT5 in cervical cancer samples.



5 MANUSCRIPT 2:

PUTATIVE BIOMARKERS FOR CERVICAL CANCER: SNVS, METHYLATION AND EXPRESSION PROFILES

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Review

Putative biomarkers for cervical cancer: SNVs, methylation and expression profiles

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ABSTRACT

Cervical cancer is primarily caused by Human papillomavirus (HPV) infection, but other factors such as smoking habits, co-infections and genetic background, can also contribute to its development. Although this cancer is avoidable, it is the fourth most frequent type of cancer in females worldwide and can only be treated with chemotherapy and radical surgery. There is a need for biomarkers that will enable early diagnosis and targeted therapy for this type of cancer. Therefore, a systems biology pipeline was applied in order to identify potential biomarkers for cervical cancer, which show significant reports in three molecular aspects: DNA sequence variants, DNA methylation pattern and alterations in mRNA/protein expression levels. CDH1, CDKN2A, RB1 and TP53 genes were selected as putative biomarkers, being involved in metastasis, cell cycle regulation and tumour suppression. Other ten genes (CDH13, FHIT, PTEN, MLH1, TP73, CDKN1A, CACNA2D2, TERT, WIF1, APC) seemed to play a role in cervical cancer, but the lack of studies prevented their inclusion as possible biomarkers. Our results highlight the importance of these genes. However, further studies should be performed to elucidate the impact of DNA sequence variants and/or epigenetic deregulation and altered expression of these genes in cervical carcinogenesis and their potential as biomarkers for cervical cancer diagnosis and prognosis.

Contents

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1. Introduction

Worldwide, there are over 527,000 new cases of cervical cancer each year, qualifying it as the fourth most frequent type of cancer among women. It is responsible for approximately 265,700 deaths per year, with the large majority occurring in less developed countries [1]. Human papillomavirus (HPV) infection is the main etiological factor for the development of cervical cancer but other aspects, such as age, smoking oral contraceptives and parity, can also contribute to its development [2].

Most HPV infections are transient and asymptomatic and the majority of HPV infections, including those with high-risk genotypes, get cleared or become undetectable within two years [3,4]. The Pap smear test is the gold standard for cervical cancer prevention, although it has low sensitivity and a high frequency of false negatives. Visual inspection with acetic acid is used as a complementary method of evaluation, but only after a positive Pap smear result [5].

The occurrence of HPV infection is related to the secretion of molecules by the innate immune system that are able to recognize the presence of HPV. These molecules show higher expression levels in the endocervical specimens infected with HPV16, assisting in avoiding the viral infection and blocking the escape of the virion from the endocytic vesicles [6,7]. However, HPV is still able to avoid the activation of the immune response by escaping from antigen presentation and downregulating pro-inflammatory signalling [8]. Additionally, the genetic background of the patient can influence the development of the disease and can be associated with different outcomes. This perspective has emphasized the importance of studying variations within the DNA sequence, alterations in epigenetic modifications and deregulation in gene expression in order to determine genetic markers [9].

These findings pave the major steps towards personalized medicine, a tool to provide more reliable approaches for diagnosis and improve prognosis. Some molecular panels have been developed for breast and colorectal cancer [10,11], but none are available for cervical cancer. This study aims to identify the main genes associated with cervical cancer and their role in carcinogenesis, thus highlighting potential biomarkers for early diagnosis, prognosis and targeted therapy.

2. Biomarker selection for cervical cancer

A systems biology pipeline was established to search the Metacore™ database for genes with variations in their DNA sequence, alterations in methylation patterns and changes in mRNA/protein expression patterns in cervical cancer samples. After that, the obtained results were cross-referenced with previously obtained data. At this point, a list of diseases affected by each molecular aspect of the selected genes in Metacore™ was collected.

Manual data mining of scientific studies using the scientific search engines PubMed and ScienceDirect was conducted to identify the genes that showed significant molecular alterations in cervical cancer, leading to the identification of a total of 2980 candidate genes. Single nucleotide variation (SNV) occurrence was studied in 2487 genes, while only 54 genes were evaluated for methylation patterns changes. The mRNA/protein levels were evaluated for 2464 genes, but the reports rarely correlated with the role of SNVs and epigenetic modifications in these genes (Fig. 1).

A cross-reference analysis was performed, allowing the identification of genes reported with the following three aspects in cervical cancer: DNA sequence variation, alteration in methylation or changes in mRNA/protein levels. A total of 14 genes were selected, among them nine were tumour suppressors genes (TP53, RB1, TP73, APC, PTEN, FHIT, CDH1, CDH13, WIF1); two cell cycle regulator genes (CDKN1A, CDKN2A); one gene related to mismatch repair (MLH1); one oncogene (TERT); and one gene encoding a calcium channel (CACNA2D2). This approach was able to identify genes that are known to be affected by HPV infection, such as TP53 and RB1, and are highly studied in cervical cancer, as well as others relatively new to this field, such as FHIT and CDH13. This set of genes was already studied in 366 diseases and, for didactic purposes, it was divided into 17 categories according to the organ/system affected. The cancer category consisted of 180 cancer types (Fig. 2).

Integration of the data for SNVs, DNA methylation and mRNA/protein expression for each gene showed different correlation levels to different diseases (Fig. 3). Although these 14 genes were studied in cervical cancer, only CDH1, CDKN2A, RB1 and TP53 were reported as statistical significant in the literature for all the three

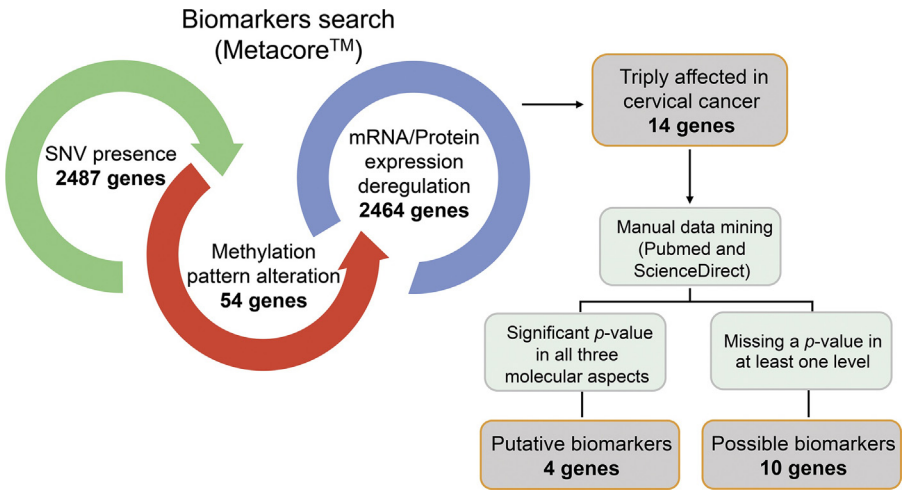

Fig. 1. Gene selection through the systems biology pipeline.

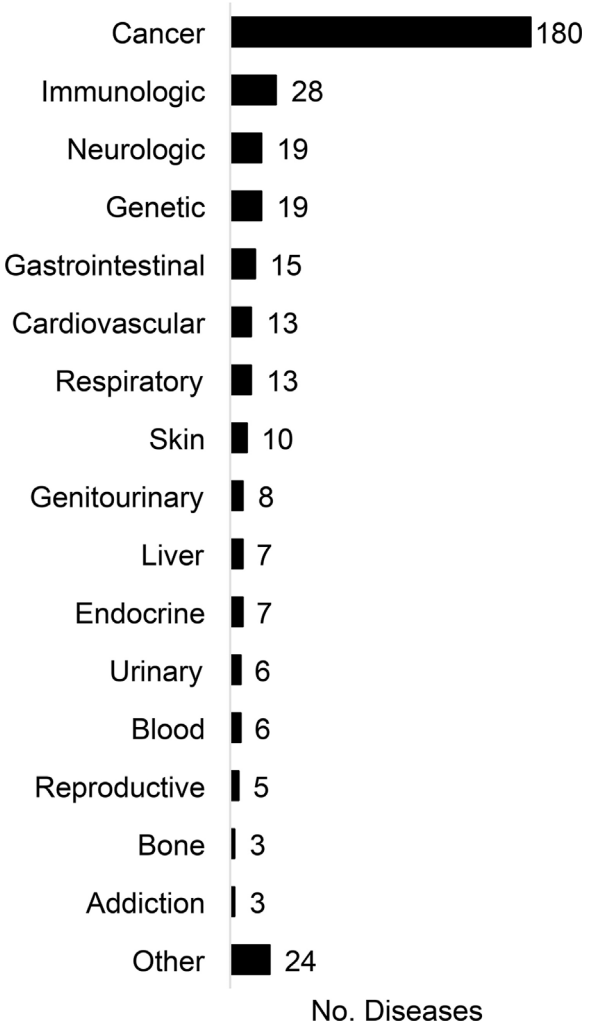

Fig. 2. Distribution of diseases observed according to the 17 Categories.

molecular aspects (Table 1), thus granting them the status of putative biomarkers.

TP53 was the most cited gene with 838 references reported in all the 17 diseases categories and 282 different diseases studied, while the CDH1 gene showed only 20 cross-References.

2.1. Variant profiles of biomarkers

Together, the CDH1, CDKN2A, RB1 and TP53 SNVs showed 494 cross-references in 79 cancer types (Fig. 4A). The list of SNVs in cervical cancer in relation to their molecular characteristics, the categories reported and the number of diseases associated with them is presented in Supplementary data, Table 1.

The CDH1 gene product, E-cadherin, is present on the membrane of epithelial cells. E-cadherin is responsible for cell adhesion, but it is also involved in transducing chemical signals inside cells, controlling cell maturation and movement, and regulating the activity of certain genes. E-cadherin also acts as a tumour suppressor [34], but only one SNV in its gene has been studied in cervical cancer. The CDH1 c.*54C > T SNV is located in the 3′-untranslated region (3′UTR), and it was identified in 280 cervical cancer samples. The T allele was shown to be protective against the development of cervical cancer ($p = 0.01$), with a lower frequency in stage III patients [12]. This SNV was also studied in 4 other cancer types (breast, oesophageal, non-small-cell lung and stomach cancers), showing significance in oesophageal carcinoma [35], and non-small-cell lung cancer [12].

The retinoblastoma 1 (RB1) gene, a tumour suppressor gene, encodes the pRB protein, which regulates cell growth and interacts with other proteins to influence cell survival, apoptosis and differentiation [36]. The RB1 c.1814 + 394G > A SNV located in the intronic region has only been studied in cervical cancer. A study on 150 samples from an Indian population showed that individuals with the AA genotype had a 1.77-fold higher risk for development of cervical cancer ($p = 0.026$) [20].

Cyclin-dependent kinase inhibitor 2A (CDKN2A) encodes two different proteins, $p14^{ARF}$ and $p16^{INK4}$, due to alternative splicing of exon 1β and exon 1α, respectively [37], and both are able to induce cell cycle arrest [38]. CDKN2A has 12 SNVs studied in cervical cancer out of which c.*29G > C located in the 3′UTR is the most reported. A study in 150 cervical cancer patients and 150 age-matched women with no malignancy demonstrated a protective effect of the G allele against cervical cancer ($p = 0.0001$). The same study showed that c.*69C > T, also located in 3′UTR, was significantly found in the patients carrying the T allele (CT or TT genotypes) ($p = 0.0004$), although the allelic frequency evaluation did not show such association ($p = 0.072$) [20]. On the contrary, no significance was observed for any of these SNVs in a study of 92 abnormal cervical samples infected with HPV16 and 32 normal samples [39], indicating that even though CDKN2A is not involved in the viral infection and proliferation, it may be important for the neoplastic process.

The tumour suppressor 53 (TP53) gene encodes for a tumour suppressor protein that plays a critical role in determining whether the DNA will be repaired or if the damaged cell will undergo apoptosis [40]. This is the most studied gene in cancer (79 cancer types) with 40 SNVs evaluated. The missense variant of TP53 c.215C > G, corresponding to codon P72R, is the most studied SNV among the cardiovascular, endocrine, gastrointestinal and genetic categories. In a Chinese study in 323 cervical cancer patients and 568 normal samples, it was found that the CG genotype is a risk factor for the development of cervical cancer ($p = 0.02$) [29], and another study found that the genotype GG is associated with cervical cancer [30]. Despite that, this TP53 variant was not found to be associated with 43 squamous cell carcinoma (SCC) and 67 cervical intraepithelial neoplasia (CIN) samples in another study [24]. The frequency of TP53 SNVs was not only associated with different populations, as it was more common in Asian populations, but also related to different cervical cancer types, with the higher variant frequency associated with adenocarcinoma (AC) than SCC [40].

2.2. Methylation profiles of biomarkers

All four genes showed hypermethylation of the promoter regions in cervical cancer, having also been studied in the other 56 different types of cancer (Fig. 4B). CDKN2A methylation was studied in 51 cancer types, while TP53 was studied in just seven types of cancer, namely cervical cancer, breast cancer, hepatocellular carcinoma, multiple myeloma, non-small-cell lung cancer, ovarian cancer and stomach cancer.

Although TP53 is the most studied gene in various cancer types, little is known about its role in cervical cancer. Only one study with 125 cervical cancer samples and 100 control samples describing the methylation of the TP53 gene in association with cancer risk ($p < 0.05$) has been reported [31]. Hypermethylation of TP53 had already been reported in other diseases such as hepatocellular carcinoma in relation to the reduced expression of TP53 [41], as well as in breast cancer [42] and ovarian cancer [43], thereby reinforcing the importance of its alteration in carcinogenesis.

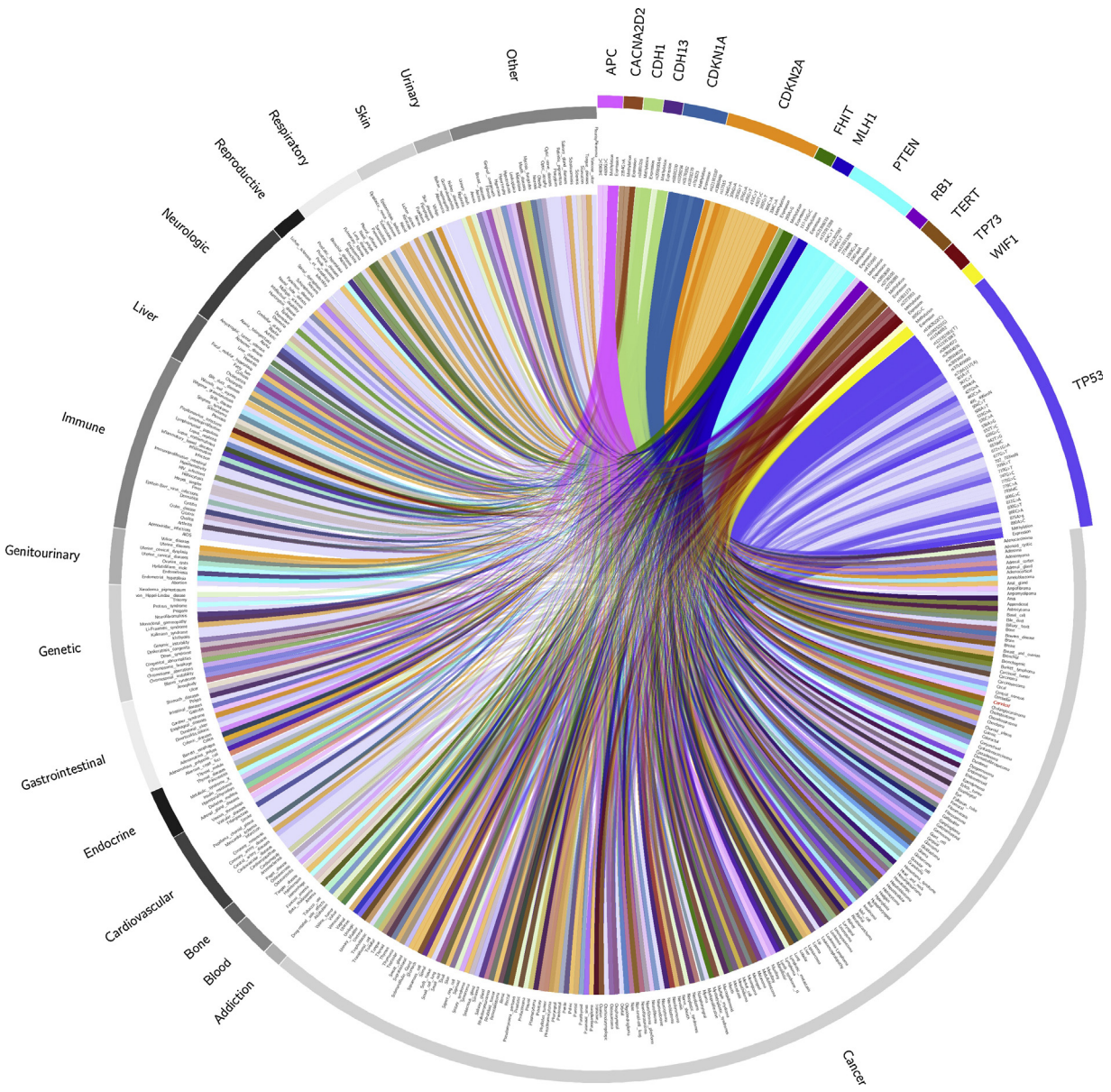

Fig. 3. Relation between SNVs, alterations in methylation patterns and deregulation of mRNA/protein expression in all the 14 selected genes and diseases within the categories. The information on the genes are organized in tabs in the outer portion of the circle. In each gene tab, the information is displayed following the sequence of SNVs, alterations in methylation patterns and deregulation of mRNA/protein expression. In the inner circle, there is a category tab showing the list of diseases that belong to a certain system/organ. Lines connect each gene characteristic to the diseases. Transparency is attributed to colour of the lines, so that opacity is directly related to the number of diseases affected by each molecular aspect.

A study investigating the methylation status of CDKN2A in 78 cervical cancer samples showed significant hypermethylation in cervical cancer in comparison with the normal cervix ($p < 0.0001$), thus also correlating the methylation status of CDKN2A to the stage of cancer [21]. The hypermethylation status of CDKN2A indeed increased with the progression from low- to high-grade lesions of the cervix ($p < 0.05$) [22], which could explain the reduced expression of $p16^{INK4}$ in the high-grade cervical lesions [44]. However, there are conflicting results for CDKN2A methylation thus questioning the significance of the methylation status being observed in the cancer and normal samples [45].

The hypermethylation of the RB1 promoter was studied in 50 samples from patients with prior lesions in the cervix and 15 cervix samples from patients with normal cytology and colposcopy. The results demonstrated an increasing degree of methylation with the severity of the lesion ($p = 0.009$) as well as an association with HPV infection ($p = 0.042$) [28]. However, these results are still controversial [31].

The CDH1 gene showed increasing methylation levels from the normal to dysplastic to invasive cervical cancer samples ($p < 0.05$) [15]. This gene was also hypermethylated in several SCC and CIN3 samples [14]. A study in 82 cervical cancer samples investigating 16 genes, including DAPK, RARB, FHIT, and TIMP3, demonstrated that CDH1 is the most hypermethylated gene and is associated with the cancer stage ($p = 0.0005$) [16]. Together with CDH13 (H-cadherin), CDH1 hypermethylation was also associated with worse disease prognosis ($p < 0.05$) [17], indicating its participation in cervical cancer. It was associated with a 92.8-fold risk of relapse ($p = 0.005$) and a 7.8-fold risk of death ($p = 0.001$) [13]. In HeLa cells, the hypermethylation of the CDH1 promoter region was associated with the absence of gene expression. However, the treatment with the green tea polyphenol (−)-epigallocatechin-3-gallate could

Table 1
List of genes and their molecular aspects reported in cervical cancer showing experimental design and *p*-value.

Gene	Molecular aspect	Samples	Sample characterization	Sample type; Country	Analysis	p-value	Ref.
CDH1	c.+54C > T	280 SG 330 C	280 CC + 330 normal cytology	Blood; China	PCR-RFLP	0.01	[12]
	Methylation	49 SG 40 C	49 CC + 40 non-malignant gynaecological diseases	Serum; Austria	MPCR	0.001	[13]
		49 SG 22 C	49 ISCC + 22 normal cytology	FFPE + LBC; USA	PS	<0.05	[14]
		62 SG 38 C	35 CC + 27 SIL + 38 normal cytology	Fresh Tissue; India	MSP	<0.05	[15]
		121 SG 8 C	77 SCC + 5 AC + 39 CIN + 8 normal cytology	Fresh tissue, FFPE, LBC; Colombia + Germany	MSP	0.0005	[16]
		93 SG	84 SCC + 9 AC	Serum; Austria	MPCR	<0.05	[17]
	Expression	100 SG 6 C	70 CC + 11 CIN3 + 19 CIN1/2 + 6 Normal	TMA; China	IHC	<0.05	[18]
		135 SG 55 C	135 SCC + 55 normal cytology	FFPE; China	IHC	<0.05	[19]
CDKN2A	c.*29G > C	150 SG 150 C	150 CC + 150 normal cytology	Fresh Tissue + LBC; India	PCR-RFLP	0.0001	[20]
	Methylation	78 SG 24 C	66 SCC + 12 AC + 24 normal cytology	Fresh frozen tissue; South Korea	MSP	<0.0001	[21]
		68 SG	23 ISCC + 10 CIN3 + 8 CIN2 + 27 CIN1	Fresh frozen tissue + LBC; USA	IIa + HTa + TBS	<0.05	[22]
	Expression	166 SG	16 SCC + 2 AC + 98 HSIL + 50 LSIL	FFPE; Brazil	IHC	<0.001	[23]
		110 SG 20 C	43 ISCC + 38 CIN3 + 11 CIN2 + 18 CIN1 + 20 normal cytology	Fresh frozen tissue; Egypt	IHC	0.01	[24]
		132 SG 17 C	20 ISCC + 20 HSIL + 42 LSIL + 50 ASCUS + 17 normal cytology	LBC; China	ICC + qRT-PCR	<0.05	[25]
		200 SG 30 C	30 SCC + 50 CIN3 + 50 CIN2 + 50 CIN1	FFPE; China	IHC	0.046	[26]
		45 SG 5 CL 48 C	5 CL + 45 HSIL + 48 normal cytology	LBC; Germany	qRT-PCR	<0.001	[27]
RB1	c.1814 + 394G > A	150 SG 150 C	150 CC + 150 normal cytology	Fresh tissue + LBC; India	PCR-RFLP	0.02	[20]
	Methylation	50 SG 15 C	18 CC + 15 HSIL + 17 LSIL + 15 normal cytology	Fresh tissue + LBC; Brazil	MSP	0.009	[28]
	Expression	110 SG 20 C	43 ISCC + 38 CIN3 + 11 CIN2 + 18 CIN1 + 20 normal cytology	Fresh frozen tissue; Egypt	IHC	<0.05	[24]
TP53	c.215C > G	328 SG 568 C	328 CC + 568 normal cytology	Blood; China	PCR-RFLP	0.02	[29]
		114 SG 200 C	103 AC + 9 SCC + 200 cancer-free cervix	Blood.; China	PCR-RFLP	0.009	[30]
	Methylation	125 SG 100 C	125 CC + 100 healthy females	Fresh tissue + Blood; India	MSP	<0.05	[31]
	Expression	110 SG 20 C	43 ISCC + 38 CIN3 + 11 CIN2 + 18 CIN1 + 20 normal cytology	Fresh frozen tissue; Egypt	IHC	0.01	[24]
		125 SG	92 SCC + 33 AC	FFPE, China	IHC	<0.05	[32]
		60 SG 60 C	60 CC + 60 normal tumour-adjacent regions	FFPE, China	IHC	<0.05	[33]

AC: Adenocarcinoma; ASCUS: atypical cells of undetermined significance; C: Control; CIN: Cervical intraepithelial neoplasia; CL: Cell line; CC: Cervical cancer; FFPE: formalin-fixed paraffin-embedded; HSIL: High-grade squamous intraepithelial lesion; HTa: HELP-tagging assay; ICC: Immunocytochemistry; IHC: Immunohistochemistry; IIa: Illumina Infinium assay; ISCC: Invasive squamous cervical carcinoma; LBC: Liquid-based cytology; LSIL: Low-grade squamous intraepithelial lesion; MPCR: MethyLight PCR; MSP: Methylation-specific PCR; PCR-RFLP: Polymerase chain reaction Restriction fragment length polymorphism; PS: Pyrosequencing; qRT-PCR: Quantitative real-time: polymerase chain reaction; SCC: Squamous cervical carcinoma; SG: study group; SIL: Squamous intraepithelial lesion; TBS: Targeted bisulfide sequencing; TMA: Tissue microarray paraffin-embedded

revert this effect [46], which suggests that the CDH1 gene can be a relevant biomarker for targeted therapy.

2.3. Expression profiles of biomarkers

Our cross-referencing results showed that a total of 394 mRNA/protein expression were associated with 169 cancer types, and studies in 33 cancer types demonstrated the deregulation of expression for all the genes (Fig. 4C). TP53 and CDKN2A, which were found to be upregulated in cervical cancer, are the most studied genes in cancer, while CDH1 and RB1 were found to be downregulated.

Deregulation of TP53 protein expression was the most reported among the four biomarkers selected and was affected in 144 cancer types. During cervical infection with high-risk HPV, the production of the oncoprotein E6 targets the p53 protein for proteosomal degradation [47], resulting in lower levels of p53 in the cervical cancer cells. Therefore, low levels of p53 have been found in the cells infected with HPV16 and producing oncoprotein E6 [48]. Another study in 125 patients showed the presence of p53 in 56.8% of the cases, with higher levels in AC compared to SCC ($p < 0.05$). The level of p53 was also associated with lymph node metastasis ($p < 0.05$) [32]. A study in 60 cervical cancer samples and 60 controls also showed higher p53 levels in the cervical cancer samples than the normal samples ($p < 0.05$), and the elevated p53 level was also associated with lymph node metastasis ($p < 0.05$) [33]. This identifies p53 as a robust prognostic biomarker in cervical cancer, even though its regulatory mechanism needs to be elucidated further.

Although CDKN2A encodes for both $p14^{ARF}$ and $p16^{INK4}$, most studies have focused on the latter. The expression of $p16^{INK4}$ has been evaluated in 114 different types of cancer, and a study in 50

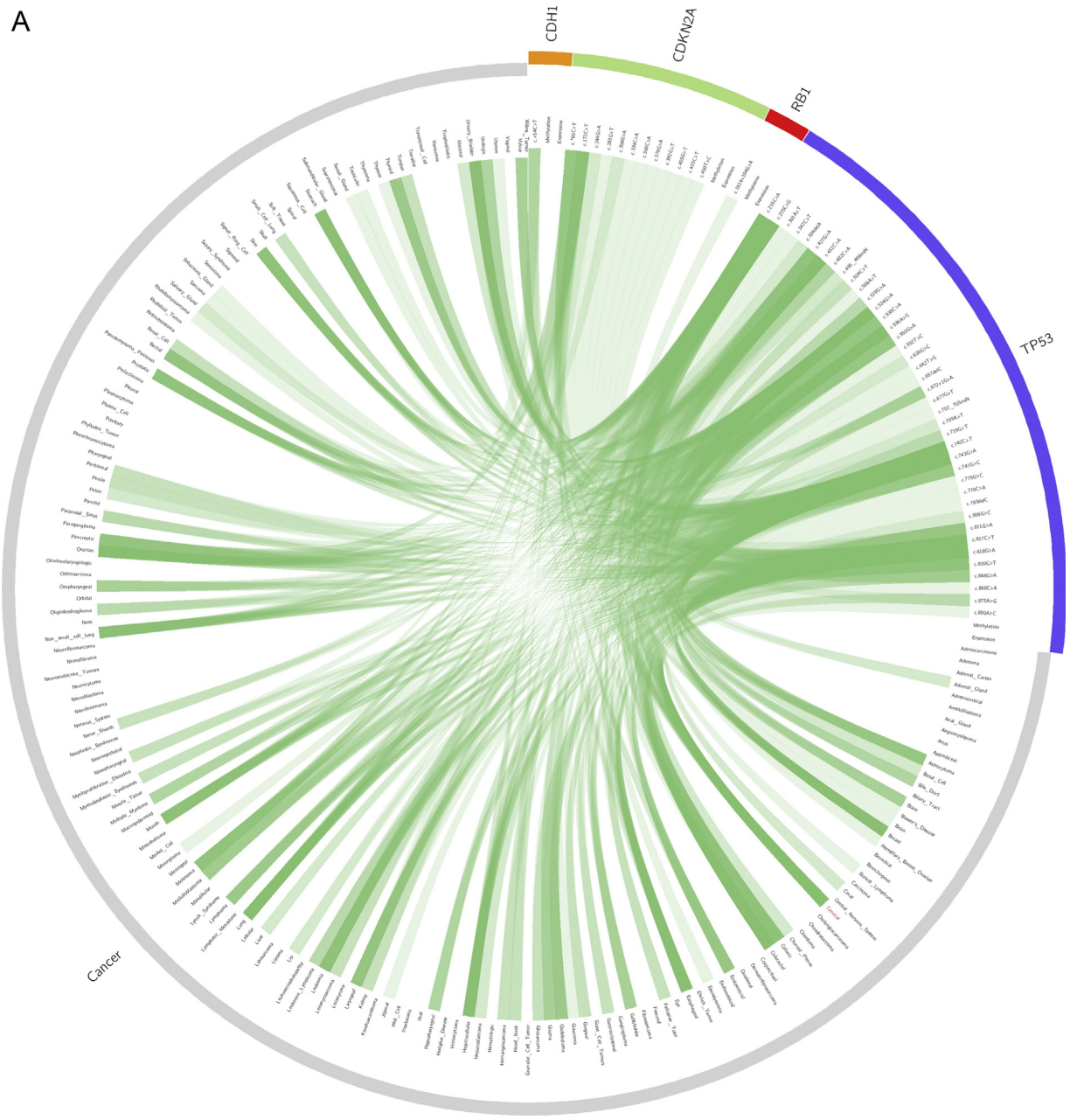

Fig. 4. Relation of molecular aspects with cancer-associated diseases for the 4 candidate biomarkers comprising (A) SNVs, (B) alterations in methylation patterns and (C) deregulation of mRNA/protein expression.

low-grade squamous intraepithelial lesions (LSIL), 98 high-grade squamous intraepithelial lesions (HSIL), 16 SCC and 2 AC. The levels of $p16^{INK4}$ increased with the severity of the cervical lesions ($p < 0.05$) [23], and similar results were obtained in 20 HSIL, 42 LSIL, 50 atypical squamous cells of undetermined significance (ASCUS), 20 cervical cancer and 17 normal samples ($p < 0.05$) [25]. Furthermore, another study with 30 normal cervix samples, 150 CIN lesions and 50 cervical cancer samples showed the over-expression of $p16^{INK4}$, which was associated with poor cervical cancer prognosis ($p < 0.05$) [26]. The levels of $p16^{INK4}$ and $p14^{ARF}$ were evaluated in 5 cervical cell lines, 48 normal samples and 48 HSIL samples, and showed a 6.27 and 4.87-fold increase, respectively, compared to the normal samples [27]. It is interesting to note that although higher $p16^{INK4}$ levels were significantly associated with the progression of cervical lesions to cervical cancer ($p = 0.001$), no CDKN2A variant was linked to the increase in protein levels [24], indicating that different types of regulation other than methylation events could be involved and should be evaluated.

Deregulation of the RB1 gene has been reported in 67 types of cancer. Similar to p53, pRB is also affected by high-risk HPV infection. In this case, upon binding of oncoprotein E7, the complex of pRB with E2F is disrupted, leading to the expression of E2F responsive genes and pRB degradation [48,49]. A study in 130 cervix samples showed decreasing levels of pRB with increasing severity of the cervical lesions ($p = 0.01$) [24], and similar results were observed in a study with 114 cervical tissue samples [50]. However, a study in 98 samples of cervical neoplastic lesions showed no alteration in the expression of pRB compared to the normal samples [51]. It has also been demonstrated that the inhibition of HPV16 E7 can restore the activity of pRB [52].

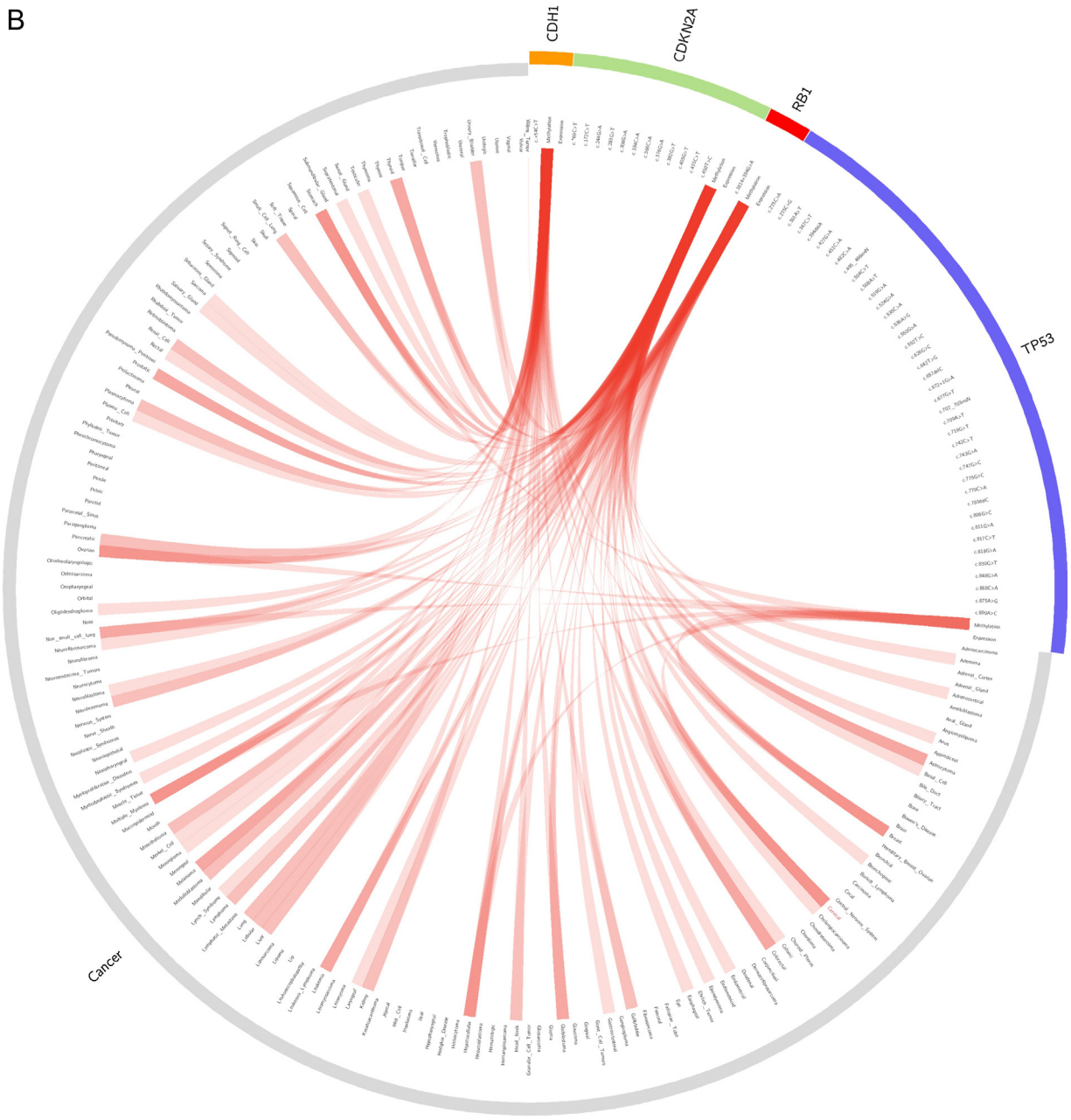

Fig. 4. (Continued)

Evaluation of CDH1 expression was performed in 60 types of cancer. For cervical cancer, a study in 40 patients with HSIL caused by HPV16 infection showed lower levels of E-cadherin upon the expression of HPV16 E6/E7 genes ($p < 0.01$) [18]. This decrease led to the promotion of cell proliferation and increase in cell migration and invasion due to weaker cell adhesion properties [53]. Additionally, reduced levels of E-cadherin and β-catenin were observed in 135 cervical cancer samples, which was associated with histological differentiation ($p < 0.001$), metastasis ($p < 0.001$) and recurrence ($p < 0.001$) [19], suggesting that the E-cadherin status could be used as prognostic biomarker.

3. Gene interactions networks

To determine the metabolic importance of CDH1, CDKN2A, RB1 and TP53 genes in the development of cervical cancer, their metabolic pathways were studied, by text and data mining of scientific papers, together with the molecules related to HPV infection. Additionally, the molecular interactions with the other 10 genes were also investigated for cervical cancer in order to assess their role in cervical carcinogenesis (Fig. 5).

3.1. Tumour suppressor pathways

The epidermal growth factor receptor (EGFR) pathway regulates cell growth, cellular maturation, proliferation, inhibition of apoptosis, angiogenesis and metastasis [54]. EGFR is known to be activated by binding to different ligands and stimulating the dimerization of the receptor. The dimerization induces the activation of the tyrosine kinase domain, which leads to auto-phosphorylation and recruitment of a range of adaptor proteins such as growth factor receptor bound protein2 (GRB2) and the proto-oncogene tyrosine protein kinase (Src). The intracellular signalling cascades affect gene transcription, which in turn

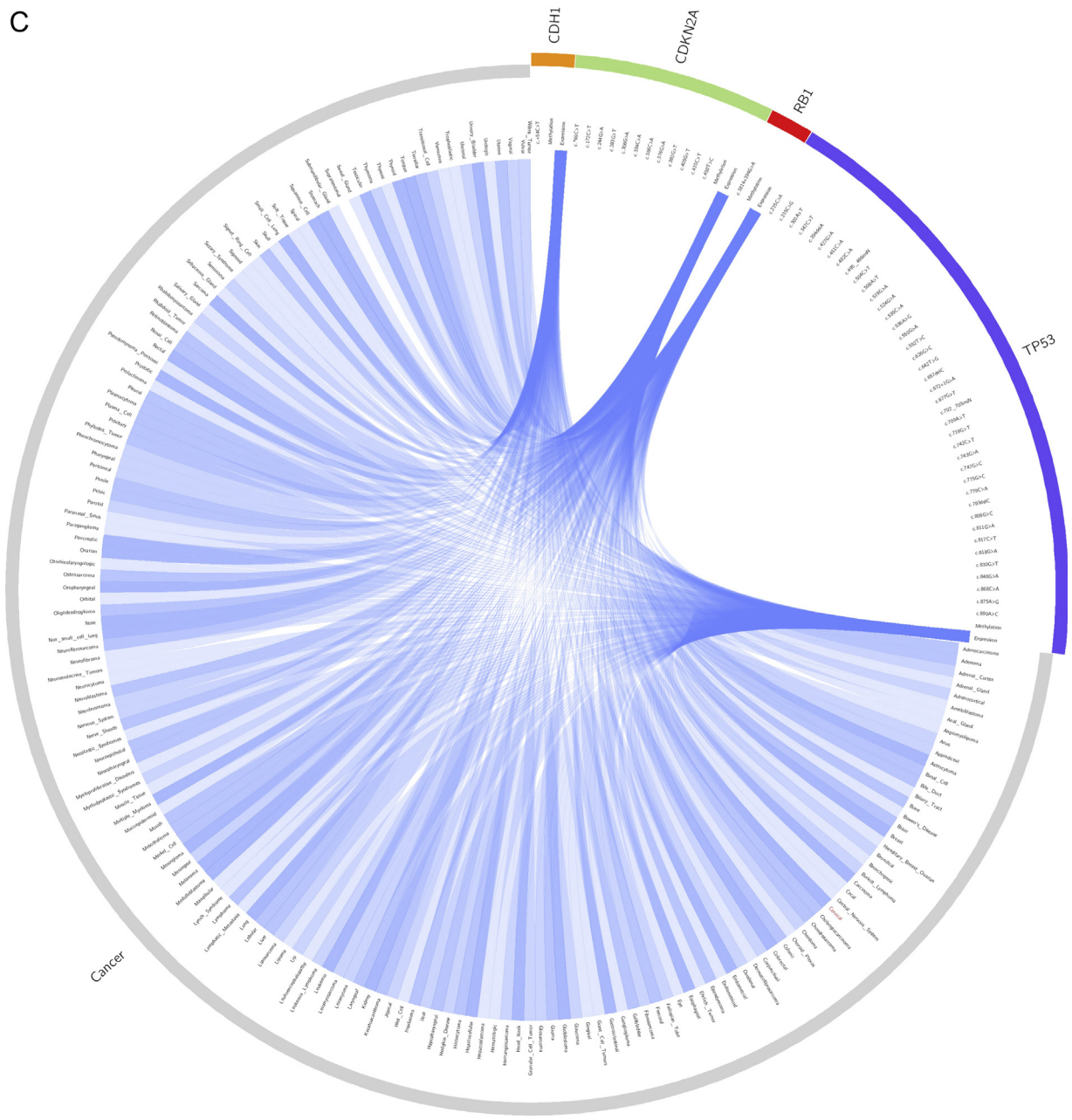

Fig. 4. (Continued)

promotes cancer cell proliferation, reduces apoptosis, increases invasion and metastasis and stimulates tumour-induced angiogenesis [55].

E-cadherin and H-cadherin (CDH13) play key roles in cell–cell adhesion, being involved in EGFR inhibition through the negative regulation of ligand binding [56,57]. Low expression of CDH1 gene can facilitate EGFR signalling, and disruptions in the function of E-cadherin leads to the enhancement of this pathway and changes in cell–cell and cell–matrix adhesion. This can increase the severity of the cervical lesions and induce metastasis [58]. The downregulation of CDH13 as a result of hypermethylation, is also associated with poor disease-free survival [17], possibly due to the lack of inhibition of the EGFR pathway. However, further analysis should be performed to elucidate the contributions of epigenetic events and gene variants on the function of the protein if the CDH13 methylation status is cannot be well-established [14,17,59]. Additionally, it is not clear how the variant allele in CDH1 c. *54C > T confers protection against cervical cancer [12].

In the EGFR pathway, Src kinase phosphorylates fragile histidine triad (FHIT), a tumour suppressor protein, to inactivate it and target it for proteasomal degradation in cancer cells. The tumour supressor properties of FHIT include the ability to induce apoptosis, arrest cell cycle and suppress tumourigenesis in nude mice [60]. High-risk HPV infections can induce FHIT loss of heterozygosity (LOH) in cervical cancer patients [61]. The epigenetic deregulation of FHIT [14,45,59,62,63] appears to be a late event during cervical carcinogenesis [64], arising from high instability in response to genotoxicities or replicative stress [65]. Smoking habits in patients with cervical cancer contribute to the homo/hemizygous deletion and downregulation of FHIT [66]. However, some variants can lead to the loss of protein function as reported in the case of the c.293A > G missense SNV at codon 35 (histidine is replaced by an arginine) or the absence of exon 5 [67].

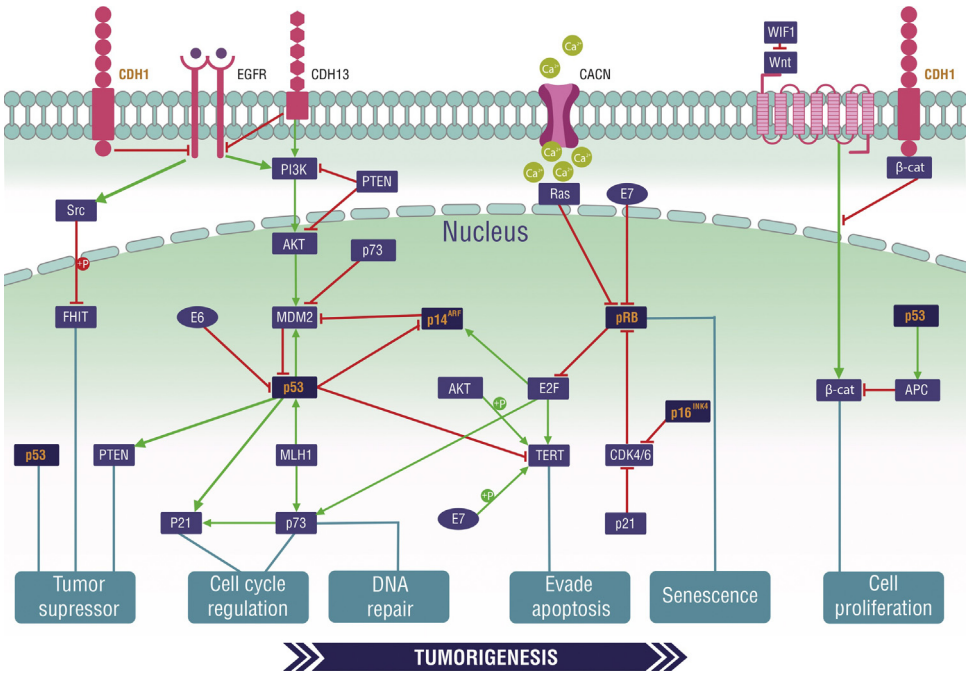

Fig. 5. Metabolic interaction networks including the four putative biomarkers and 10 possible biomarkers and their cellular outcomes.

Together with low levels of FHIT, elevated levels of p53 are related a higher recurrence of cervical cancer [68] resulting in worse prognosis. The tumour suppressor properties of TP53 result from its antiproliferative functions as it can induce G1 arrest (by functioning as a cell-cycle checkpoint), senescence (by causing permanent cell cycle arrest) and modulate autophagy [69]. Allelic imbalance in TP53 was shown to be associated with a reduced relapse-free survival [70], reinforcing its importance in the maintenance of cancer and indicating it as a putative predictor of poor prognosis.

3.2. Cell cycle regulation and DNA repair

Cell cycle dysregulation is one of the main mechanisms by which cancers develops. The ability to sustain proliferation in response to continuous growth-stimulatory signals is a hallmark of cancer development, translating into the evasion from growth suppressors [71]. The phosphatidylinositol 3-kinase (PI3K) pathway is one of the most relevant pathways in cancer due to its role in apoptosis prevention, cellular survival and proliferation promotion. Activation of the PI3K pathway is able to stimulate transmembrane tyrosine kinase receptors, such as EGFR, promoting its dimerization and leading to the phosphorylation of PtdIns $(4,5)P_2$ (PIP2) into PtdIns$(3,4,5)P_3$ (PIP3), which acts as second messenger in the cell [72]. PIP3 is the principal mediator of the PI3K pathway, and protein kinase B (AKT) is one of the most important downstream effectors, which can activate other important pathways, such as the rapamycin complex 1 (mTORC1) [73]. The PI3K signalling pathway can be inhibited by phosphatase and tensin-homologue (PTEN) through the conversion of PIP3 into PIP2. Similar to p53, PTEN has several tumour suppressor properties and can affect cellular functions such as survival, proliferation, energy metabolism and cellular architecture [74].

Regulation of PTEN expression can occur at several levels. In cervical cancer, PTEN variants were shown to be associated with HPV-negative adenocarcinoma [75], although it was not reproduced in other studies [76]. The epigenetic regulation of gene expression seems to be relevant for PTEN as it is hypermethylated in cervical cancer and associated with the disease stage [77] and histological type [78], which is consistent with lower levels of PTEN and PTEN silencing [76,79]. The methylation events were also reported to be affected by the SNV in the CpG islands c.-9C > G, which modifies the methylation status and thus gene expression [80]. PTEN c.273delA, located in the functional domain of PTEN can interfere with the function of PTEN [81], showing the importance of the loss of function of PTEN in cancer development.

The p53 protein, a well-known tumour suppressor, is located downstream of the PI3K pathway and is tightly regulated by the proto-oncogene MDM2 (MDM2), $p14^{ARF}$, p73 and MutL homolog 1 (MLH1). It stimulates the expression of p21 to arrest cell cycle and its inactivation through the oncoprotein effects of HPV-E6 on the cell cycle leads to a cascade of deregulating events that induces cell proliferation. p53 is regulated by cyclin dependent kinase inhibitor 1A (CDKN1A), which is also activated by p73 (TAp73). p21 acts as regulator of cell cycle and inhibits the activity of CDK2, which is required for the phosphorylation of pRB [82]. Thus, it is important to determine the impact of variants in this gene. In Chinese and in non-Asian women, the missense variant c.93C > A located in codon 31 was associated with protective effects against cervical cancer [83,84]. In the Brazilian population, c.93C > A and c.*20C > T (located in 3′UTR) variants of CDKN1A were also associated with protection against cervical cancer [85]. CDKN1A was found to be hypermethylated in less than 10% of cervical cancer samples [31]. This is consistent with the reports of high levels of p21 in cervical cancer samples, which was also shown to be associated with the progression of lesions in several studies [23,24,86,87].

The gene encoding tumour suppressor 73 (TP73) is a functional and structural homologue of p53 that codes for multiple proteins through alternative splicing. There are mainly two isoforms of this gene: TAp73, which is a full length protein containing a transactivation domain (TA) that is able to activate other genes and induce apoptosis and ΔNp73, the NH2-terminal truncated isoform that acts as an inhibitor of TAp73 [88]. Epigenetic deregulation of TP73 is dependent on the production of high-risk HPV E6 and E7 oncoproteins leading to cellular immortalization [89]. This gene is also hypermethylated to a higher degree in AC than in SCC. Hypermethylation in the promoter region of TP73 not only increases the risk of cervical cancer by 1.81-fold [31] but is

also associated with lower expression levels of the protein. Furthermore, the TAp73 gene was found to be overexpressed in radiosensitive cervical cancer cells, and ΔNp73 overexpression was correlated with resistance to radiation and worse outcome [88], thus indicating the epigenetic and expression deregulation of TP73 as potential prognostic biomarkers in cervical carcinogenesis. Although there is a lack of association of SNVs in NearGene-5 of TP73 (c.-20G > A and c.-30C > T) with cervical cancer [90], its presence may have consequences in the methylation status and expression of TP73.

The regulation of these proteins is very tight. Increased p73 levels were found to be associated with higher levels of the p21 protein [91]. Both p53 and p73 proteins can be stimulated by the MLH1 protein, thereby affecting the cell cycle regulation. MLH1 also participates in intracellular processes for recognizing and repairing foreign DNA, by a process called DNA mismatch repair. Sequence variants in a set of proteins involved in this process, including MLH1, have been shown to increase the susceptibility to cancer due to the high levels of microsatellite instability [92]. Hypermethylation of the MLH1 promoter has been reported in cervical cancer [93] and is associated with low-risk HPV infection [94]. Significant lower levels of MLH1 has been found in invasive cervical cancer (ICC) samples [95], suggesting that the loss of MLH1 protein is associated with neoplastic invasiveness [96]. Reduced levels of MLH1, FHIT together with increased levels of p53 proteins have been considered indicators of higher recurrence of cervical cancer [68], which could be an indicative of their potential as candidates for cervical cancer prognosis.

3.3. Senescence and apoptosis evasion pathways

Calcium voltage-gated channel auxiliary subunit alpha2 delta2 (CACNA2D2) is a member of the calcium channel family, CACN. The input of Ca^{2+} into the cytoplasm is one of the mechanisms by which the RAS pathway is activated [97]. There are very few studies investigating the role of CACNA2D2 in cervical cancer but its presence in a locus deleted in 15% of cervical cancer samples suggests that it could be a tumour suppressor gene [98]. In fact, the CACNA2D2 loci was found to be deleted in 50% of cervical cancer samples or its increased methylation level, which ranged from 9% to 27%, was shown to promote the progression from CIN to cervical cancer, resulting in over 5 times reduction in CACNA2D2 expression in cervical cancer samples in comparison with normal samples [99].

The RAS pathway leads to cell growth, differentiation and survival once the Ras protein inhibits pRb. pRB is maintained in an active hypophosphorylated state by $p16^{INK4}$ and is able to repress the E2F-mediated transcription, inducing cell cycle arrest and senescence [100]. The downregulation of pRb is related to the activation of p53 by increasing levels of E2F, thereby stimulating $p14^{ARF}$. $p14^{ARF}$ inhibits MDM2 [101], an important negative regulator of p53 and pRB, which promotes proliferation by stimulating the S phase through the induction of the transcriptional activity of E2F1/DP1. AKT and E2F are responsible for phosphorylation and activation of telomerase reverse transcriptase (TERT), an oncogene responsible for telomere elongation that allows the cells to evade apoptosis [102]. HPV16 E6 binds to the TERT promoter and induces its expression, leading to significant higher levels of TERT in the cervical cancer samples [103,104] as well as the upregulation of vascular endothelial growth factor (VEGF) [105], which contributes for tumour angiogenesis. Silencing of TERT leads to the suppression of cell proliferation, cell cycle, cell migration and invasion leading to the induction of apoptosis, thereby suppressing the growth of cervical cancer cells in vitro [106]. However, hypermethylation of the TERT promoter was found to be significant in CIN3 lesions [107] and cervical cancer samples [108]. TERT promoter variants c.-146C > T and c.-124C > T were observed in 21.4% of cervical cancer patients in India, which is almost 4 times higher than the proportions reported by studies in Western populations [109].

3.4. Cell proliferation

The Wnt signalling pathway is a major regulator of cell proliferation, migration, differentiation and tumour progression [110] and is important for cervical cancer formation as it is affected by several genes included in this study, such as CDH1, Wnt inhibitory factor 1 (WIF1) and adenomatous polyposis coli (APC). The Wnt pathway plays a key role in cell proliferation via the transcriptional activation by β-catenin mediated through the binding of E-cadherin. On the other hand, WIF1 inhibits ligation of Wnt to its receptor frizzled, leading to the activation of the Axin complex and constant degradation of β-catenin [110], which prevents signalling by the Wnt pathway. This has been implicated in the inhibition of tumour proliferation, invasion, angiogenesis and apoptosis [111]. Hypermethylation of WIF1 was detected in approximately 87% of the cervical cancer samples [14,112], which could be correlated to low levels or absence of WIF1 protein in the cervical cancer samples [111].

APC, another negative regulator of Wnt pathway, is involved not only in the Axin complex but also in actin assembly, cell-to-cell adhesion and microtubule network formation [113]. APC hypermethylation was found in cancer cell lines but was absent in HPV-immortalized cell lines [114], indicating that genetic silencing due to methylation may be a later event in transformation or might be cell cycle dependent [114]. In cervical cancer samples, APC hypermethylation was significantly higher in AC compared to SCC [16,115]. Low levels of APC in the cytoplasm was correlated with high levels of β-catenin in the nucleus and cytoplasm of cervical cancer cells, suggesting the activation of the Wnt pathway [116]. In HeLa and CaSki cells, treatment with hydralazine promotes APC demethylation, inducing the expression of APC and leading to growth inhibition [117]. This shows the importance of the Wnt signalling pathway in the development of cervical cancer and denotes WIF1 and APC as therapeutic targets in cervical cancer.

4. Conclusion

The process of finding compelling biomarkers for the development or prognosis of cervical cancer is complex. In this study, it was possible to unveil four putative biomarkers that are significantly correlated with the development and maintenance of cervical cancer due to the presence of variants in the respective genes, alterations in gene methylation patterns and deregulated expression patterns. Further studies should be performed to elucidate the role of these four genes as well as to include the others ten possible genes that lack studies with significance due to the inconsistency of the number of patients and chosen methodology to determine the presence of SNV, methylation status and expression deregulation in these genes. This will allow the development of a robust genetic panel for the early diagnosis and evaluation of prognosis of cervical cancer as has been already observed for other types of cancers.

Conflict of interest statement

The authors declare that there are no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mrrev.2017.06.002.

References

[1] J. Ferlay, I. Soerjomataram, M. Ervik, R. Dikshit, S. Eser, C. Mathers, M. Rebelo, D. Parkin, D. Forman, F. Bray, GLOBOCAN 2012 v1.0, cancer incidence and mortality worldwide: IARC CancerBase No. 11, lyon, Fr. Int. Agency Res. Cancer (2012) 2012. http://globocan.iarc.fr (Accessed 28 October 2016).
[2] S.V. Bava, A.K.T. Thulasidasan, C.N. Sreekanth, Cervical cancer: a comprehensive approach towards extermination, Ann. Med. 48 (2016) 149–161, doi:http://dx.doi.org/10.3109/07853890.2016.1145796.
[3] J.W. Kim, S.H. Song, C.H. Jin, J.K. Lee, N.W. Lee, K.W. Lee, Factors affecting the clearance of high-risk human papillomavirus infection and the progression of cervical intraepithelial neoplasia, J. Int. Med. Res. 40 (2012) 486–496.
[4] M. Molano, A. Van den Brule, M. Plummer, E. Weiderpass, H. Posso, A. Arslan, Meijer C.J.L.M, N. Muñoz, S. Franceschi, Determinants of clearance of human papillomavirus infections in Colombian women with normal cytology: a population-based, 5-year follow-up study, Am. J. Epidemiol. 158 (2003) 486–494, doi:http://dx.doi.org/10.1093/aje/kwg171.
[5] L.A. Torre, R.L. Siegel, E.M. Ward, A. Jemal, Global cancer incidence and mortality rates and trends — an update, Cancer Epidemiol. Biomarkers Prev. 25 (2016) 16–27, doi:http://dx.doi.org/10.1158/1055-9965 (EPI-15-0578).
[6] I.I. Daud, M.E. Scott, Y. Ma, S. Shiboski, S. Farhat, A.B. Moscicki, Association between toll-like receptor expression and human papillomavirus type 16 persistence, Int. J. Cancer 128 (2011) 879–886, doi:http://dx.doi.org/10.1002/ijc.25400.
[7] C.B. Buck, P.M. Day, C.D. Thompson, J. Lubkowski, W. Lu, D.R. Lowy, J.T. Schiller, Human alpha-defensins block papillomavirus infection, Proc. Natl. Acad. Sci. U. S. A. 103 (2006) 1516–1521, doi:http://dx.doi.org/10.1073/pnas.0508033103.
[8] I.H. Frazer, Interaction of human papillomaviruses with the host immune system: a well evolved relationship, Virology 384 (2009) 410–414, doi:http://dx.doi.org/10.1016/j.virol.2008.10.004.
[9] C. on A.I.A.S.B. and G.F. in Health, Genes, Behavior, and the social environment, The National Academies Press, 2006.
[10] C. Bellcross, W.D. Dotson, Tumor gene expression profiling in women with breast cancer, PLoS Curr. 2 (2010) RRN1178, doi:http://dx.doi.org/10.1371/currents.RRN1178.
[11] D. Cragun, C. Radford, J.S. Dolinsky, M. Caldwell, E. Chao, T. Pal, Panel-based testing for inherited colorectal cancer: a descriptive study of clinical testing performed by a US laboratory, Clin. Genet. 86 (2014) 510–520, doi:http://dx.doi.org/10.1111/cge.12359.
[12] Y. Li, Y. Tang, R. Zhou, D. Sun, Y. Duan, N. Wang, Z. Chen, N. Shen, Genetic polymorphism in the 3′-untranslated region of the E-cadherin gene is associated with risk of different cancers, Mol. Carcinog. 50 (2011) 857–862, doi:http://dx.doi.org/10.1002/mc.20765.
[13] R. Abudukadeer, G. Bakry, A. Mutz-Dehbalaie, G.K. Widschwendter, H. Fiegl, Clinical relevance of CDH1 and CDH13 DNA-methylation in serum of cervical cancer patients, Int. J. Mol. Sci. 13 (2012) 8353–8363, doi:http://dx.doi.org/10.3390/ijms13078353.
[14] E.M. Siegel, B.M. Riggs, A.L. Delmas, A. Koch, A. Hakam, K.D. Brown, Quantitative DNA methylation analysis of candidate genes in cervical cancer, PLoS One 10 (2015) e0122495, doi:http://dx.doi.org/10.1371/journal.pone.0122495.
[15] S. Pathak, N. Bhatla, N. Singh, Cervical cancer pathogenesis is associated with one-carbon metabolism, Mol. Cell. Biochem. 369 (2012) 1–7, doi:http://dx.doi.org/10.1007/s11010-012-1362-3.
[16] G. Narayan, H. Arias-pulido, S. Koul, H. Vargas, F.F. Zhang, J. Villella, A. Schneider, M.B. Terry, M. Mansukhani, V.V. Murty, Frequent promoter methylation of CDH1 DAPK, RARB, and HIC1 genes in carcinoma of cervix uteri: its relationship to clinical outcome, Mol. Cancer 2 (2003) 24.
[17] L. Widschwendter, A. Ivarsson, H. Müller, A. Fiegl, G. Müller-Holzner, C. Goebel, M. Marth, CDH1 and CDH13 methylation in serum is an independent prognostic marker in cervical cancer patients, Int. J. Cancer 109 (2004) 163–166, doi:http://dx.doi.org/10.1002/ijc.11706.
[18] Y. Liu, W. Qian, J. Zhang, Y. Dong, C. Shi, Z. Liu, S. Wu, The indicative function of Twist2 and E-cadherin in HPV oncogene-induced epithelial-mesenchymal transition of cervical cancer cells, Oncol. Rep. 33 (2015) 639–650, doi:http://dx.doi.org/10.3892/or.2014.3620.
[19] Y. Cheng, Y. Zhou, W. Jiang, X. Yang, J. Zhu, D. Feng, Y. Wei, M. Li, F. Yao, W. Hu, W. Xiao, B. Ling, Significance of E-cadherin, β-catenin, and vimentin expression as postoperative prognosis indicators in cervical squamous cell carcinoma, Hum. Pathol. 43 (2012) 1213–1220, doi:http://dx.doi.org/10.1016/j.humpath.2011.08.025.
[20] N. Thakur, S. Hussain, V. Nasare, B.C. Das, S.F. Basir, M. Bharadwaj, Association analysis of p16 (CDKN2A) and RB1 polymorphisms with susceptibility to cervical cancer in Indian population, Mol. Biol. Rep. 39 (2012) 407–414, doi:http://dx.doi.org/10.1007/s11033-011-0752-z.
[21] M.Y. D.H. Jeong, Y.N. Youm, K.B. Kim, M.S. Lee, H.K. Sung, K.T. Kim Yoon, Promoter methylation of p16, DAPK, CDH1, and TIMP-3 genes in cervical cancer: correlation with clinicopathologic characteristics, Int. J. Gynecol. Cancer (2006) 1234–1240.
[22] N.A. Wijetunga, T.J. Belbin, R.D. Burk, K. Whitney, M. Abadi, J.M. Greally, M.H. Einstein, N.F. Schlecht, Gynecologic Oncology Novel epigenetic changes in CDKN2A are associated with progression of cervical intraepithelial neoplasia, Gynecol. Oncol. 142 (2016) 566–573, doi:http://dx.doi.org/10.1016/j.ygyno.2016.07.006.
[23] E.A. Portari, B. Russomano, M.J. Camargo, C.R.M. Gayer, M.J. Guillobel, C.B. Santos-rebouças, J.M.B. Macedo, Immunohistochemical expression of cyclin D1, p16Ink4a, p21WAF1, and Ki-67 correlates with the severity of cervical neoplasia, Int. J. Gynecol. Pathol. 32 (2013) 501–508, doi:http://dx.doi.org/10.1097/PGP.0b013e31826f5cf6.
[24] A.A. Bahnassy, A.R.N. Zekri, M. Saleh, M. Lotayef, M. Moneir, O. Shawki, The possible role of cell cycle regulators in multistep process of HPV-associated cervical carcinoma, BMC Clin. Pathol. 7 (2007) 4, doi:http://dx.doi.org/10.1186/1472-6890-7-4.
[25] C. Zhang, W.E.I. Bao, L. Wang, Downregulation of p16INK4A inhibits cell proliferation and induces G1 cell cycle arrest in cervical cancer cells, Int. J. Mol. Med. 33 (2014) 1577–1585, doi:http://dx.doi.org/10.3892/ijmm.2014.1731.
[26] H. Liu, Y. Wang, L. Wang, M. Hao, P16INK4A and survivin. Diagnostic and prognostic markers in cervical intraepithelial neoplasia and cervical squamous cell carcinoma, Exp. Mol. Pathol. 99 (2015) 44–49, doi:http://dx.doi.org/10.1016/j.yexmp.2015.04.004.
[27] H. Von Keyserling, W. Ku, A. Schneider, T. Bergmann, A.M. Kaufmann, p16 INK4a and p14 ARF mRNA expression in Pap smears is age-related, Mod. Pathol. 25 (2012) 465–470, doi:http://dx.doi.org/10.1038/modpathol.2011.179.
[28] T.M. McCormick, N.H.S. Canedo, Y.L. Furtado, F.A. Silveira, R.J. de Lima, A.D.F. Rosman, G.L. Almeida Filho, M. da G. da C. Carvalho, Association between human papillomavirus and Epstein – Barr virus DNA and gene promoter methylation of RB1 and CDH1 in the cervical lesions: a transversal study, Diagn. Pathol. 10 (2015) 59, doi:http://dx.doi.org/10.1186/s13000-015-0283-3.
[29] R. F. Yuan, P. Sun, Y. Chen, S. Ni Liang, Combined analysis of pri-miR-34b/c rs4938723 and TP 53 Arg72Pro with cervical cancer risk, Tumor Biol. (2016) 6267–6273, doi:http://dx.doi.org/10.1007/s13277-015-4467-y.
[30] S. Yang, Y. Cai, P. Jiang, W. Li, J. Tang, Association of a miR-502-Binding site single nucleotide polymorphism in the 3 ' – Untranslated region of SET8 and the TP53 codon 72 polymorphism with cervical cancer in the chinese population, Asian Pac. J. Cancer Prev. 15 (2014) 6505–6510.
[31] M. Jha, V. Nikbakht, A. Jain, N. Sehgal, Promoter hypermethylation of p73 and p53 genes in cervical cancer patients among north Indian population, Mol. Biol. Rep. 39 (2012) 9145–9157, doi:http://dx.doi.org/10.1007/s11033-012-1787-5.
[32] S. Cai, K. Han, Research on expression and importance of p53, p16 and VEGF-C in cervical cancer, J. Gynecol. Obstet. Biol. La Reprod. 44 (2015) 639–645, doi:http://dx.doi.org/10.1016/j.jgyn.2014.07.012.
[33] L. Liu, X. Li, H. Chen, J. Cui, D. Xu, Significance of Ebp1 and p53 protein expression in cervical cancer, Genet. Mol. Res. 14 (2015) 11860–11866, doi:http://dx.doi.org/10.4238/2015.October.2.19.
[34] F. van Roy, G. Berx, The cell–cell adhesion molecule E-cadherin, Cell. Mol. Life Sci. 65 (2008) 3756–3788, doi:http://dx.doi.org/10.1007/s00018-008-8281-1.
[35] Y.M. X.F. Zhang, H. Wang, Y.Y. Ge, Z.F. Cao, D.G. Chen, W. Wen, N. Guo, Y. Wang, J.H. Zhang Li, Association of CDH1 single nucleotide polymorphisms with susceptibility to esophageal squamous cell carcinomas and gastric cardia carcinomas, Dis. Esophagus 21 (2008) 21–29, doi:http://dx.doi.org/10.1111/j.1442-2050..00724.x.
[36] C. Giacinti, A. Giordano, RB and cell cycle progression, Oncogene 25 (2006) 5220–5227, doi:http://dx.doi.org/10.1038/sj.onc.1209615.
[37] L. Mao, A. Merlo, G. Bedi, G.I. Shapiro, C.D. Edwards, B.J. Rollins, D. Sidransky, A novel pl6INK4A transcript, Cancer Res. 55 (1995) 2995–2997.
[38] H. Kanao, T. Enomoto, Y. Ueda, M. Fujita, R. Nakashima, Y. Ueno, T. Miyatake, T. Yoshizaki, G.S. Buzard, T. Kimura, K. Yoshino, Y. Murata, Correlation between p14(ARF)/p16(INK4A) expression and HPV infection in uterine cervical cancer, Cancer Lett. 213 (2004) 31–37, doi:http://dx.doi.org/10.1016/j.canlet.2004.03.030.
[39] A. Chansaenroj, P. Theamboonlers, S. Junyangdikul, A. Swangvaree, T. Karalak, Polymorphisms in TP53 (rs1042522), p16 (rs11515 and rs3088440) and NQO1 (rs1800566) genes in Thai cervical cancer patients with HPV 16 infection, Asian Pac. J. Cancer Prev. 14 (2013) 341–346. http://www.ncbi.nlm.nih.gov/pubmed/23534750.
[40] M.L. Tornesello, L. Buonaguro, F.M. Buonaguro, Mutations of the TP53 gene in adenocarcinoma and squamous cell carcinoma of the cervix: a systematic

review, Gynecol. Oncol. 128 (2013) 442–448, doi:http://dx.doi.org/10.1016/j.ygyno.2012.11.017.
[41] I.P. Pogribny, S.J. James, Reduction of p53 gene expression in human primary hepatocellular carcinoma is associated with promoter region methylation without coding region mutation, Cancer Lett. 176 (2002) 169–174, doi:http://dx.doi.org/10.1016/S0304-3835(01)00748-0.
[42] J.H. Kang, S.J. Kim, D.-Y. Noh, I.A. Park, K.J. Choe, O.J. Yoo, H.-S. Kang, Methylation in the p53 promoter is a supplementary route to breast carcinogenesis: correlation between CpG methylation in the p53 promoter and the mutation of the p53 gene in the progression from ductal carcinoma in situ to invasive ductal carcinoma, Lab. Investig. 81 (2001) 573–579, doi:http://dx.doi.org/10.1038/labinvest.3780266.
[43] M. Chmelarova, E. Krepinska, J. Spacek, J. Laco, M. Beranek, V. Palicka, Methylation in the p53 promoter in epithelial ovarian cancer, Clin. Transl. Oncol. 15 (2012) 160–163, doi:http://dx.doi.org/10.1007/s12094-012-0894-z.
[44] G.J. Nuovo, T.W. Plaia, S.A. Belinsky, S.B. Baylin, J.G. Herman, In situ detection of the hypermethylation-induced inactivation of the p16 gene as an early event in oncogenesis, PNAS 96 (1999) 12754–12759.
[45] C. Banzai, K. Nishino, J. Quan, K. Yoshihara, M. Sekine, T. Yahata, K. Tanaka, Promoter methylation of DAPK1, FHIT, MGMT, and CDKN2A genes in cervical carcinoma, Int. J. Clin. Oncol. 19 (2014) 127–132, doi:http://dx.doi.org/10.1007/s10147-013-0530-0.
[46] M.A. Khan, A. Hussain, M.K. Sundaram, U. Alalami, (−) −Epigallocatechin-3-gallate reverses the expression of various tumor-suppressor genes by inhibiting DNA methyltransferases and histone deacetylases in human cervical cancer cells, Oncol. Rep. 33 (2015) 1976–1984, doi:http://dx.doi.org/10.3892/or.2015.3802.
[47] B. Tummers, S.H. Van Der Burg, High-risk human papillomavirus targets crossroads in immune signaling, Viruses 7 (2015) 2485–2506, doi:http://dx.doi.org/10.3390/v7052485.
[48] S. Shukla, S. Mahata, G. Shishodia, A. Pandey, A. Tyagi, Functional regulatory role of STAT3 in HPV16-Mediated cervical carcinogenesis, PLoS One 8 (2013) e67849, doi:http://dx.doi.org/10.1371/journal.pone.0067849.
[49] C.A. Moody, L.A. Laimins, Human papillomavirus oncoproteins: pathways to transformation, Nat. Rev. Cancer 10 (2010) 550–560, doi:http://dx.doi.org/10.1038/nrc2886.
[50] B. Tringler, C.J. Gup, M. Singh, S. Groshong, A.L. Shroyer, D.E. Heinz, K.R. Shroyer, Evaluation of p16INK4a and pRb expression in cervical squamous and glandular neoplasia, Hum. Pathol. 35 (2004) 689–696, doi:http://dx.doi.org/10.1016/j.humpath.2004.02.012.
[51] T. Sano, K. Oyama, T. Kashiwabara, Immunohistochemical overexpression of p16 protein associated with intact retinoblastoma protein expression in cervical cancer and cervical intraepithelial neoplasia, Pathol. Int. (1998) 580–585.
[52] C. Guo, K. Liu, H. Luo, H. Chen, Y. Zheng, S. Sun, Q. Zhang, L. Huang, Potent anti-tumor effect generated by a novel human papillomavirus (HPV) antagonist peptide reactivating the pRb/E2F pathway, PLoS One 6 (2011) e17734, doi:http://dx.doi.org/10.1371/journal.pone.0017734.
[53] D. Hu, J. Zhou, F. Wang, H. Shi, Y. Li, HPV-16 E6/E7 promotes cell migration and invasion in cervical cancer via regulating cadherin switch in vitro and in vivo, Arch. Gynecol. Obstet. 292 (2015) 1345–1354, doi:http://dx.doi.org/10.1007/s00404-015-3787-x.
[54] C. Yewale, D. Baradia, I. Vhora, S. Patil, A. Misra, Epidermal growth factor receptor targeting in cancer: a review of trends and strategies, Biomaterials 34 (2013) 8690–8707, doi:http://dx.doi.org/10.1016/j.biomaterials.2013.07.100.
[55] N. Normanno, A. De Luca, C. Bianco, L. Strizzi, M. Mancino, M.R. Maiello, A. Carotenuto, G. De Feo, F. Caponigro, D.S. Salomon, Epidermal growth factor receptor (EGFR) signaling in cancer, Gene 366 (2006) 2–16, doi:http://dx.doi.org/10.1016/j.gene.2005.10.018.
[56] G.-Y. Bae, S.-J. Choi, J.-S. Lee, J. Jo, J. Lee, J. Kim, H.-J. Cha, Loss of E-cadherin activates EGFR-MEK/ERK signaling, which promotes invasion via the ZEB1/MMP2 axis in non-small cell lung cancer, Oncotarget 4 (2013) 2512–2522.
[57] E. Kyriakakis, K. Maslova, M. Philippova, D. Pfaff, M.B. Joshi, S.A. Buechner, P. Erne, T.J. Resink, T-Cadherin is an auxiliary negative regulator of EGFR pathway activity in cutaneous squamous cell carcinoma: impact on cell motility, J. Invest. Dermatol. 132 (2012) 2275–2285, doi:http://dx.doi.org/10.1038/jid.2012.131.
[58] G. Christofori, Changing neighbours, changing behaviour: cell adhesion molecule-mediated signalling during tumour progression, EMBO J. 22 (2003) 2318–2323.
[59] Q. Feng, A. Balasubramanian, S.E. Hawes, P. Toure, S. Sow, A. Dem, B. Dembele, C.W. Critchlow, L. Xi, H. Lu, M.W. Mcintosh, A.M. Young, N.B. Kiviat, Detection of hypermethylated genes in women with and without cervical neoplasia, J. Natl. Cancer Inst. 97 (2005) 273–282, doi:http://dx.doi.org/10.1093/jnci/dji041.
[60] F. Bianchi, A. Magnifico, C. Olgiati, N. Zanesi, Y. Pekarsky, E. Tagliabue, C.M. Croce, S. Mé Nard, M. Campiglio, P.K. Vogt, FHIT-proteasome degradation caused by mitogenic stimulation of the EGF receptor family in cancer cells, PNAS 103 (2006) 18981–18986.
[61] D. Butler, C. Collins, M. Mabruk, C.B. Walsh, M.B. Leader, E.W. Kay, Deletion of the FHIT gene in neoplastic and invasive cervical lesions is related to high-risk HPV infection but is independent of histopathological features, J. Pathol. 192 (2000) 502–510.
[62] D. Butler, C. Collins, M. Mabruk, M.B. Leader, E.W. Kay, Loss of Fhit expression as a potential marker of malignant progression in preinvasive squamous cervical cancer, Gynecol. Oncol. 86 (2002) 144–149, doi:http://dx.doi.org/10.1006/gyno.2002.6712.
[63] K. Ki, S. Lee, S. Tong, J. Lee, D. Song, S. Chi, Role of 5 â€™ −CpG island hypermethylation of the FHIT gene in cervical carcinoma, J. Gynecol. Oncol. 19 (2008) 117–122, doi:http://dx.doi.org/10.3802/jgo.2008.19.2.117.
[64] A.K. Virmani, C. Muller, A. Rathi, S. Zoechbauer-mueller, M. Mathis, A.F. Gazdar, Aberrant methylation during cervical carcinogenesis, Clin. Cancer Res. 7 (2001) 584–589.
[65] C.E. Waters, J.C. Saldivar, S.A. Hosseini, K. Huebner, The FHIT gene product: tumor suppressor and genome caretaker, Celllular Mol, Life Sci. 71 (2015) 4577–4587, doi:http://dx.doi.org/10.1007/s00018-014-1722-0.
[66] C.H. Holschneider, R.L. Baldwin, K. Tumber, C. Aoyama, B.Y. Karlan, Human cancer biology the fragile histidine triad gene: a molecular link between cigarette smoking and cervical cancer, Hum. Cancer Biol. 11 (2005) 5756–5763, doi:http://dx.doi.org/10.1158/1078-0432 (CCR-05-0234).
[67] M.K. Neyaz, S. Hussain, M.I. Hassan, B.C. Das, S.A. Husain, M. Bharadwaj, Novel missense mutation in FHIT gene: interpreting the effect in HPV-mediated cervical cancer in Indian women, Mol. Cell. Biochem. 335 (2010) 53–58, doi:http://dx.doi.org/10.1007/s11010-009-0240-0.
[68] A.A. Bahnassy, A.R.N. Zekri, D. Ph, M.S. Madbouly, The correlation between FHIT, P53 and MMR genes in human papillomavirus-associated cervical carcinoma, J. Egiptian Nat. Cancer. Inst. 18 (2006) 191–202.
[69] J.T. Zilfou, S.W. Lowe, Tumor suppressive functions of p53, cold spring harb, Perspect. Biol. 0 (2009) 1001883, doi:http://dx.doi.org/10.1101/cshperspect.a001883.
[70] Å.H. Elland, S.M.K. Raggerud, G.B.K. Ristensen, R.H. Olm, V.M.A. Beler, K.H. Uebner, A.B.Ø. Ale, R.A.L. Othe, Primary cervical carcinomas show 2 common regions of deletions at 3P, 1 within the FHIT gene: evaluation of allelic inbalance at FHIT, RB1 and TP53 in relation to survival, Int. J. Cancer. 88 (2000) 217–222.
[71] D. Hanahan, R.A. Weinberg, Hallmarks of cancer: the next generation, Cell 144 (2011) 646–674, doi:http://dx.doi.org/10.1016/j.cell.2011.02.013.
[72] J. Polivka, F. Janku, Molecular targets for cancer therapy in the PI3 K/AKT/mTOR pathway, Pharmacol. Ther. 142 (2014) 164–175, doi:http://dx.doi.org/10.1016/j.pharmthera.2013.12.004.
[73] M. MArtini, Ma.C. de Santis, L. Braccini, F. Gulluni, E. Hirsch, PI3K/AKT signaling pathway and cancer: an updated review, Ann. Med. 46 (2014) 372–383.
[74] M.S. Song, L. Salmena, P.P. Pandolfi, The functions and regulation of the PTEN tumour suppressor, Nat. Rev. Mol. Cell Biol. 13 (2012) 283–296, doi:http://dx.doi.org/10.1038/nrm3330.
[75] T. Minaguchi, H. Yoshikawa, S. Nakagawa, T. Yasugi, T. Yano, H. Iwase, K. Mizutani, K. Shiromizu, K. Ohmi, Y. Watanabe, K. Noda, M. Nishiu, Y. Nakamura, Y. Taketani, Association of PTEN mutation with HPV-negative adenocarcinoma of the uterine cervix, Cancer Lett. 210 (2004) 57–62, doi:http://dx.doi.org/10.1016/j.canlet.2004.03.017.
[76] T.-H. Cheung, K.W.-K. Lo, S.-F. Yim, L.K.-Y. Chan, M.-S. Heung, C.-S. Chan, A.Y.-K. Cheung, T.K.-H. Chung, Y.-F. Wong, Epigenetic and genetic alternation of PTEN in cervical neoplasm, Gynecol. Oncol. 93 (2004) 621–627, doi:http://dx.doi.org/10.1016/j.ygyno.2004.03.013.
[77] Q. Qi, Y. Ling, M. Zhu, L. Zhou, M. Wan, Y. Bao, Y. Liu, Promoter region methylation and loss of protein expression of PTEN and significance in cervical cancer, Biomed. Rep. 2 (2014) 653–658, doi:http://dx.doi.org/10.3892/br.2014.298.
[78] H.-J. Yang, V.W.S. Liu, Y. Wang, P.C.K. Tsang, H.Y.S. Ngan, Differential DNA methylation profiles in gynecological cancers and correlation with clinico-pathological data, BMC Cancer 6 (2006) 212, doi:http://dx.doi.org/10.1186/1471-2407-6-212.
[79] M.G. Eijsink, M. a ten Hoor, H. Kok, H.W. de Bock, E. Nijman, G.B. a Wisman, A. G.J. van der Zee, The epidermal growth factor receptor pathway in relation to pelvic lymph node metastasis and survival in early-stage cervical cancer, Hum. Pathol. 41 (2010) 1735–1741, doi:http://dx.doi.org/10.1016/j.humpath.2010.04.017.
[80] Y. Harima, S. Sawada, K. Nagata, M. Sougawa, V. Ostapenko, T. Ohnishi, Mutation of the PTEN gene in advanced cervical cancer correlated with tumor progression and poor outcome after radiotherapy, Int. J. Oncol. 18 (2001) 493–497.
[81] M.M.A. Rizvi, M.S. Alam, A. Ali, S.J. Mehdi, S. Batra, a K. Mandal, Aberrant promoter methylation and inactivation of PTEN gene in cervical carcinoma from Indian population, J. Cancer Res. Clin. Oncol. 137 (2011) 1255–1262, doi:http://dx.doi.org/10.1007/s00432-011-0994-0.
[82] A. Psyrri, R.A. Defilippis, A.P.B. Edwards, K.E. Yates, L. Manuelidis, D. Dimaio, Role of the retinoblastoma pathway in renescence triggered by repression of the human papillomavirus E7 protein in cervical carcinoma cells, Cancer Res. 64 (2004) 3079–3086.
[83] N. Wang, S. Wang, Q. Zhang, Y. Lu, H. Wei, W. Li, S. Zhang, D. Yin, Y. Ou, Association of p21 SNPs and risk of cervical cancer among Chinese women, BMC Cancer 12 (2012) 589, doi:http://dx.doi.org/10.1186/1471-2407-12-589.
[84] Y. Ma, Y. Zhang, L. Lin, X. Guo, Quantitative assessment of the relationship between p21 Ser31Arg polymorphism and cervical cancer, Tumor Biol. 34 (2013) 3887–3892, doi:http://dx.doi.org/10.1007/s13277-013-0976-8.
[85] S.L. Vargas-torres, E.A. Portari, A.L. Silva, Roles of CDKN1A gene polymorphisms (rs1801270 and rs1059234) in the development of cervical neoplasia, Tumor Biol. 37 (2016) 10469–10478, doi:http://dx.doi.org/10.1007/s13277-016-4850-3.

[86] K. Ishida, A. Araki, An evaluation of the diagnostic and prognostic significance immunostaining in squamous intraepithelial lesions of the uterine cervix using liquid-based cytology specimens, Diagn. Cytopathol. 42 (2013) 125–133, doi:http://dx.doi.org/10.1002/dc.
[87] Y. Zhang, L. Guo, P. Xing, Y. Chen, F. Li, W. Zhu, X. Lu, Increased expression of oncogene-induced senescence markers during cervical squamous cell cancer development, Int. J. Clin. Exp. Pathol. 7 (2014) 8911–8916.
[88] S.S. Liu, K. Chan, A.N. Cheung, X. Liao, T. Leung, H. Ngan, Expression of #Np73 and TAp73 an independently associated with radiosensitivities and prognoses in cervical squamous cell carcinoma, Clin. Cancer Res. 73 (2006) 3922–3927, doi:http://dx.doi.org/10.1158/1078-0432 (CCR-05-2573).
[89] F.E. Henken, S.M. Wilting, R.M. Overmeer, J.G.I. van Rietschoten, a O.H. Nygren, A. Errami, J.P. Schouten, Meijer C.J.L.M, P.J.F. Snijders, R.D.M. Steenbergen, Sequential gene promoter methylation during HPV-induced cervical carcinogenesis, Br. J. Cancer 97 (2007) 1457–1464, doi:http://dx.doi.org/10.1038/sj.bjc.6604055.
[90] S.S. Wang, M.C. Bratti, A.C. Rodríguez, R. Herrero, R.D. Burk, C. Porras, P. González, M.E. Sherman, Z.E. Lan, M. Schiffman, S.J. Chanock, Common variants in immune and DNA Rrepair genes and risk for human papillomavirus persistence and progression to cervical cancer, J. Infect. Dis. 199 (2009) 20–30, doi:http://dx.doi.org/10.1086/595563 (Common.).
[91] S.S. Liu, R.C. Leung, K.Y. Chan, P. Chiu, A.N. Cheung, K. Tam, T. Ng, L. Wong, H.Y. Ngan, p73 expression is associated with the cellular radiosensitivity in cervical cancer after radiotherapy, Clin. Cancer Res. 10 (2004) 3309–3316.
[92] A.R. Ellison, J. Lofing, G.A. Bitter, Human MutL homolog (MLH1) function in DNA mismatch repair: a prospective screen for missense mutations in the ATPase domain, Nucleic Acids Res. 32 (2004) 5321–5338, doi:http://dx.doi.org/10.1093/nar/gkh855.
[93] S. Sood, F.D. Patel, S. Ghosh, A. Arora, L.K. Dhaliwal, R. Srinivasan, Epigenetic alteration by DNA methylation of ESR1, MYOD1 and hTERT gene promoters is useful for prediction of response in patients of locally advanced invasive cervical carcinoma treated by chemoradiation, Clin. Oncol. (R. Coll. Radiol.) 27 (2015) 720–727, doi:http://dx.doi.org/10.1016/j.clon.2015.08.001.
[94] A. Spathis, E. Aga, M. Alepaki, A. Chranioti, C. Meristoudis, I. Panayiotides, D. Kassanos, P. Karakitsos, Promoter methylation of p16(INK4A), hMLH1, and MGMT in liquid-based cervical cytology samples compared with clinicopathological findings and HPV presence, Infect. Dis. Obstet. Gynecol. 2011 (2011) 927861, doi:http://dx.doi.org/10.1155/2011/927861.
[95] E. Giarnieri, R. Mancini, T. Pisani, M. Alderisio, A. Vecchione, Mlh1 Msh2, p53 Fhit, Bcl-2, and bax expression in invasive and in situ squamous cell carcinoma of the uterine cervix 1, Clin. Cancer Res. 6 (2000) 3600–3606.
[96] A. Ciavattini, M. Piccioni, A. Luigi Tranquilli, A. Filosa, T. Pieramici, G. Goteri, Immunohistochemical expression of DNA mismatch repair (MMR) system proteins (hMLH1, hMSH2) in cervical preinvasive and invasive lesions, Pathol. Res. Pract. 201 (2005) 21–25, doi:http://dx.doi.org/10.1016/j.prp.2004.09.012.
[97] S. Yoshiki, R. Matsunaga-Udagawa, K. Aoki, Y. Kamioka, E. Kiyokawa, M. Matsuda, Ras and calcium signaling pathways converge at Raf1 via the Shoc2 scaffold protein, Mol. Biol. Cell. 21 (2010) 1088–1096, doi:http://dx.doi.org/10.1091/mbc.E09.
[98] V. Senchenko, J. Liu, E. Braga, N. Mazurenko, W. Loginov, Y. Seryogin, I. Bazov, A. Protopopov, F.L. Kisseljov, V. Kashuba, M.I. Lerman, G. Klein, E.R. Zabarovsky, Deletion mapping using quantitative real-time PCR identifies two distinct 3p21.3 regions affected in most cervical carcinomas, Oncogene 22 (2003) 2984–2992, doi:http://dx.doi.org/10.1038/sj.onc.1206429.
[99] S. Mitra, D.M. Indra, P.S. Basu, R.K. Mondal, A. Roy, S. Roychoudhury, C.K. Panda, Alterations of RASSF1A in premalignant cervical lesions: clinical and prognostic significance, Mol. Carconigenes 51 (2012) 723–733, doi:http://dx.doi.org/10.1002/mc.20837.
[100] J.P. Williams, T. Stewart, B. Li, R. Mulloy, D. Dimova, M. Classon, The retinoblastoma protein is required for Ras-induced oncogenic transformation, Mol. Cell. Biol. 26 (2006) 1170–1182.
[101] J. Campisi, F. d'Adda di Fagagna, Cellular senescence: when bad things happen to good cells, Nat. Rev. Mol. Cell Biol. 8 (2007) 729–740, doi:http://dx.doi.org/10.1038/nrm2233.
[102] S. Jagadeesh, P.P. Banerjee, Inositol hexaphosphate represses telomerase activity and translocates TERT from the nucleus in mouse and human prostate cancer cells via the deactivation of Akt and PKCα, Biochem. Biophys. Res. Commun. 349 (2006) 1361–1367, doi:http://dx.doi.org/10.1016/j.bbrc.2006.09.002.
[103] K. Van Doorslaer, R.D. Burk, Association between hTERT activation by HPV E6 proteins and oncogenic risk, Virology 433 (2012) 216–219, doi:http://dx.doi.org/10.1016/j.virol.2012.08.006.
[104] H.-Y. Wang, S. Park, S. Kim, D. Lee, G. Kim, Y. Kim, K.H. Park, H. Lee, Use of hTERT and HPV E6/E7 mRNA RT-qPCR TaqMan assays in combination for diagnosing high-grade cervical lesions and malignant tumors, Am. J. Clin. Pathol. 143 (2015) 344–351. (Accessed 21 January 2016) http://ajcp.oxfordjournals.org/content/ajcpath/143/3/344.full.pdf.
[105] F. Li, J. Cui, Human telomerase reverse transcriptase regulates vascular endothelial growth factor expression via human papillomavirus oncogene E7 in HPV-18-positive cervical cancer cells, Med. Oncol. 32 (2015) 199, doi:http://dx.doi.org/10.1007/s12032-015-0649-0.
[106] Y. Shi, Q. Zhao, L. Zhang, W.E.I. Du, Knockdown of hTERT by siRNA inhibits cervical cancer cell growth in vitro and in vivo, Int. J. Oncol. 45 (2014) 1216–1224, doi:http://dx.doi.org/10.3892/ijo.2014.2493.
[107] N. Vasiljevic, D. Scibior-bentkowska, A.R. Brentnall, J. Cuzick, A.T. Lorincz, Gynecologic Oncology Credentialing of DNA methylation assays for human genes as diagnostic biomarkers of cervical intraepithelial neoplasia in high-risk HPV positive women, Gynecol. Oncol. 132 (2014) 709–714, doi:http://dx.doi.org/10.1016/j.ygyno.2014.02.001.
[108] N. Milutin, I. Sabol, P. Planini, G. Grubi, I. Fistoni, Methylated host cell gene promoters and human papillomavirus type 16 and 18 predicting cervical lesions and cancer, PLoS One 10 (2015) e0129452, doi:http://dx.doi.org/10.1371/journal.pone.0129452.
[109] V. Vinothkumar, G. Arunkumar, S. Revathidevi, K. Arun, M. Manikandan, A. Kuha, D. Magendhra, K.S. Rajkumar, C. Ajay, R. Rajaraman, R. Ramani, A.K. Murugan, A.K. Munirajan, TERT promoter hot spot mutations are frequent in Indian cervical and oral squamous cell carcinomas, Tumor Biol. 37 (2016) 7907–7913, doi:http://dx.doi.org/10.1007/s13277-015-4694-2.
[110] B.T. Macdonald, K. Tamai, X. He, Review Wnt/b -Catenin signaling: components, mechanisms, and diseases, Dev. Cell. 17 (2009) 9–26, doi:http://dx.doi.org/10.1016/j.devcel.2009.06.016.
[111] I. Ramachandran, E. Thavathiru, S. Ramalingam, G. Natarajan, W.K. Mills, D.M. Benbrook, R. Zuna, S. Lightfoot, A. Reis, S. Anant, L. Queimado, Wnt inhibitory factor 1 induces apoptosis and inhibits cervical cancer growth, invasion and angiogenesis in vivo, Oncogene 31 (2011) 2725–2737, doi:http://dx.doi.org/10.1038/onc.2011.455.
[112] W.F. van der Meide, S. Snellenberg, Meijer C.J.L.M, A. Baalbergen, T.J.M. Helmerhorst, W.B. van der Sluis, P.J.F. Snijders, R.D.M. Steenbergen, Promoter methylation analysis of WNT/β-catenin signaling pathway regulators to detect adenocarcinoma or its precursor lesion of the cervix, Gynecol. Oncol. 123 (2011) 116–122, doi:http://dx.doi.org/10.1016/j.ygyno.2011.06.015.
[113] I. Näthke, Cytoskeleton out of the cupboard: colon cancer and cytoskeletal changes induced by loss of APC, Nat. Rev. Cancer 6 (2006) 967–974.
[114] J.M. Kooter, S.M. Wilting, C.J.L.M. Meijer, W. Quint, D.M. Sch, P.J.F. Snijders, R. D.M. Steenbergen, Longitudinal assessment of DNA methylation changes during HPVE6E7-induced immortalization of primary keratinocytes, Epigenetics 10 (2015) 73–81.
[115] S.M. Dong, H. Kim, S. Rha, Promoter hypermethylation of multiple genes in carcinoma of the uterine cervix, Clin. Cancer Res. 7 (2001) 1982–1986.
[116] P.O. Jawanjal, S.U. Salhan, I.N. Dhawan, R.I. Tripathi, G.A. Rath, Peptidyl-prolyl isomerase Pin1-mediated abrogation of APC −β −catenin interaction in squamous cell carcinoma of cervix, Rom. J. Morphol. Embryol. 55 (2014) 83–90.
[117] Y. Song, C. Zhang, Hydralazine inhibits human cervical cancer cell growth in vitro in association with APC demethylation and re-expression, Cancer Chemother. Pharmacol. 63 (2009) 605–613.

6 MANUSCRIPT 3:

TARGETED THERAPY IN CANCER: COMPUTATIONAL ANALYSIS OF SNVS CONSEQUENCES IN VEGFR-2 AND EGFR

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Targeted therapy in cancer: computational analysis of SNVs consequences in VEGFR-2 and EGFR

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Abstract

Cancer is a major burden worldwide and it is estimated 17 million cases of cancer in the world in 2020. The choice of the therapy to be used in treatment should take into consideration each patient genetic background. In this study, missense variants were selected from VEGF, VEGFR-2 and EGFR, as these molecules are major targets in cancer treatment. The deleterious potential of each single nucleotide variant (SNV) was assessed using 16 algorithms from dbNSFP and validated using text and data mining in PubMed. Swiss-model was used to model the protein structures validated in MolProbity. The mCSM and MAESTRO algorithms were used to predict stability alteration. None of the three SNVs in VEGF were found to influence therapy choice. Two mutations with score 15 of VEGFR-2 were included as they are pivotal residues for tyrosine-kinase activity: phosphorylation site Y1059 and ATP binding residue V848. For EGFR, it was found 4 SNVs with score 16 and 23 SNVs with score 15. From these, 14 were selected for further studies as they are connected to therapeutic outcomes. The SNVs L858R, responsible for increased sensitivity to tyrosine-kinase inhibitors (TKI), K745R which is associated to absent TK activity, and G428D which leads to 100-fold weaker binding to EGFR were some of the variants included in the study. This work could help perceive the impact that SNVs could have in cancer target therapy, showing the importance of further studies to better understand the mechanisms behind the drug-target interactions.

Introduction

Cancer is a major burden worldwide and it is predicted that by 2020 there will be over 17 million new cases of cancer (Ferlay et al., 2012). Unfortunately, there are only few molecules used as drug targets, like VEGF, EGFR and VEGFR-2. These molecules have monoclonal antibodies available for cancer treatment (bezacizumab, ramucirumab, and cetuximab, respectively), once they block their signaling pathways mainly involved in angiogenesis and proliferation (Clarke and Hurwitz, 2014; Dassonville et al., 2007; Wang et al., 2004).

The vascular endothelial growth factor (VEGF) family formed by seven molecules: VEGF-A (simply VEGF), placenta growth factor (PlGF), VEGF-B, VEGF-C, VEGF-D, VEGF-E and VEGF-F. They are mitogen and survival factors for vascular endothelial cells that promote monocyte motility, vasodilatation and increased vascular permeability (Roskoski, 2008). The gene encoding VEGF is composed by 8 exons and alternative splicing leads to several isoforms (Ferrara et al., 2004) formed by 121, 145, 148, 165, 183, 189 or 206 amino acids (Hoeben et al., 2004). The incorporation of exon 8a ($VEGF_{XXX}$ - CDKPRR) or exon 8b ($VEGF_{XXX}$b SLTRKD) in the splicing step results in different isoforms (Delcombel et al., 2013; Gu et al., 2013). The VEGF-165a and -121a are the most abundant pro-angiogenic isoforms, while VEGF-165b and -121b are the most frequent anti-angiogenic ones (Delcombel et al., 2013). Therefore, the blockage of VEGF became an important strategy to treat cancer, avoiding its binding to the VEGF receptors VEGFR-1, VEGFR-2, and VEGFR-3 and two non-protein kinase co-receptors (neuropilin 1 and 2) (Roskoski, 2008). Bevacizumab is a mAb that neutralizes VEGF angiogenic properties (Ferrara et al., 2004), approved for treating advanced cervical cancer (Rodriguez-Freixinos and Mackay, 2015), non-small cell lung

cancer (Klein and Loewenstein, 2015), metastatic renal cell carcinoma and colorectal cancer (Ferrara and Adamis, 2016).

The vascular endothelial growth factor receptors (VEGFRs) are tyrosine kinase receptors (TKRs) composed by 3 domains: extracellular, with seven immunoglobulin-like domains; a transmembrane domain; and an intracellular protein-kinase domain. |This family is composed by VEGFR-1/Flt1, VEGFR-2/KDR and VEGFR-3/Flt4 (Roskoski, 2008). The binding of a growth factor in the VEGFR ectodomain leads to receptor dimerization, protein kinase activation, and initiation of signaling pathways (Roskoski, 2008). VEGFR-2 exhibits stronger kinase activity than VEGFR-1, although the lower affinity with the ligand (Roskoski, 2008), becoming a target in cancer therapy. Ramubirumab is a competitive mAb that binds to extracellular domain 3 of VEGFR-2, blocking the signaling cascade (Calvetti et al., 2015; Hofheinz and Lorenzen, 2015).

Epidermal growth factor receptor (EGFR) family, Human Epidermal Growth Factor Receptor (HER) has four members: EGFR/HER1/ErbB1, HER2/ErbB2, HER3/ErbB3 and HER4/ErbB4 that are all structurally similar (Capdevila et al., 2009; Levitzki and Klein, 2010; Maria et al., 2008). EGFR is coded by 237,600 base pair (bp) gene with 28 exons located in locus 7p11.2. The transcript has 5,616 bp and codes a 1,210 amino acids (aa) protein precursor, which results in a mature with 1,186 aa. EGFR is divided into three segments: an extracellular domain (ectodomain), a transmembrane domain, and a tyrosine kinase domain (intracellular). The ectodomain comprises four domains: the domains I and III (cysteine rich) are responsible for ligand-binding; and domain II is the dimerization arm of EGFR, directly involved in receptor dimerization (Capdevila et al., 2009; Roskoski, 2014). So, Epidermal growth factor (EGF) binds to EGFR for promoting dimerization of EGFR, inducing auto-phosphorylation and activating two signaling pathways: phosphatidylinositol 3-kinase

(PI3K)/AKT pathway and RAS-RAF-mitogen-activated protein kinase (MAPK). These two pathways are involved in cell migration, angiogenesis, inhibition of apoptosis and cell growth; all processes related to tumor development and progression (Nakai et al., 2016; Roskoski, 2014). EGFR overexpression was observed in lung, ovarian, cervical, breast, colorectal, and other cancer types (Akhtar et al. 2014; Bellone et al. 2007; Gadducci, Elena, and Greco 2013; Lee et al. 2004), thus becoming a target for developing anti-cancer therapies. There are two different approaches: use of monoclonal antibodies (mAbs) - cetuximab, matuzumab and panitumumab (Capdevila et al., 2009) through competitive inhibition that leads to EGFR internalization and consequent degradation (Nakai et al., 2016); or use of small molecules (gefitinib, erlotinib, afatinib and osimertinib) that target the cytoplasmic protein-kinase domain, binding to its ATP-binding site and inhibiting EGFR signaling (Nakai et al., 2016).

In recent years it has aroused the importance of computational studies for predictive biology, allowing the selection of putative biomarkers to cancer development (Cardoso et al., 2017), and the structural and functional impacts of DNA sequence variants in drug usage (Borba et al., 2016). Thus, the objective of this work was to evaluate the impact of missense single nucleotide variants (SNVs) of EGFR, VEGFR-2 and VEGF over the protein structure and function, and through computational approaches, to highlight therapeutic actions in cancer treatment.

1. Methods

1.1. Variants selection

The transcripts, NCBI Reference Sequences and UniProt identification was collected using Hugo Gene Nomenclature Committee (http://www.genenames.org/). The transcripts identifications used were ENST00000611736.4 for VEGF (RefSeq:

NM_001025366, NP_001020537; UniProt: P15692), ENST00000263923.4 for VEGFR-2 (RefSeq: NM_002253, NP_002244; UniProt: P35968) and ENST00000275493.6 for EGFR (RefSeq: NM_055228, NP_005219; UniProt: P00533).

1.2. SNVs scores

The variants were selected using dbNSFP (Database for Nonsynonymous SNPs' Functional Predictions) v.3.4a, a non-synonimous SNVs database for dbNSP (Single Nucleotide Polymorphism database) version 147 (Liu et al., 2016; X. Liu et al., 2011). A total of 16 algorithms for functional and structural prediction of variants impact were SIFT, Polyphen 2, LRT, Mutation Taster, Mutation Assessor, FATHMM-MKL, PROVEAN, Meta SVM, Meta LR, M-CAP and fathmm-MKL algorithms. A binary system was applied to the algorithms output according to threshold of each one, attributing score 1 for deleterious impact, and score 0 for neutral impact of the variant over the protein structure and function (Table 1). The sum of the scores obtained in each algorithm was used to select the potential SNVs for evaluating the tridimensional (3D) models. Then, manual data- and text-mining were performed in PubMed (www.ncbi.nlm.nih.gov/pubmed) to determine the therapeutic potential of the top mutations of each gene for further analysis.

1.3. Proteins structure modelling

The models for EGFR and VEGFR-2 protein structures were built using SWISS-MODEL server (https://swissmodel.expasy.org/). The PDB ID 4AGD was used as template for the TKR domain of VEGFR-2; while 1MOX and 4I23 were used for EGFR furin-like/receptor L-domain, and TKR domain, respectively. All models were validated in MolProbity (Chen et al., 2010) prior to generate the mutant structures. The

visualization of the original and mutated 3D molecular structures were performed in PyMol (https://www.pymol.org/), a molecular visualization tool system. In order to access the variants stability impact in each protein, it was used mCSM (Pires et al., 2014) and MAESTRO (Laimer et al., 2015) prediction algorithms, both based in the protein's three dimensional structure previously modelled.

2. Results

2.1. SNVs selection and scores

The algorithm evaluation showed higher number of variants in the receptors, reaching to 542 in VEGFR-2 and 521 in EGFR, while only 116 variants were listed for VEGF. The Weibull score distribution of SNVs for the three genes is present in Figure 1, showing the presence of a right tale for all genes (with shape lower than 2.6). The models used for VEGFR-2 TK domain, EGFR furin-like/receptor L-domain, and TKR domain obtained a score of 2.17, 1.57 and 1.33 in MolProbity, respectively.

VEGF showed no variant in the highest score, and only three variants C266Y, V258M and R262Q reached to score 14. However, none of these mutations were found associated with therapeutic impact, so VEGF was excluded of the structural analysis.

The VEGFR-2 showed two variants achieving a score of 16, L902Q and Y1100F, but they could not be associated to therapeutic drugs. So, other five SNVs found in score 15 were evaluated, and V848E and Y1059C were reported in literature as possibly influencing therapeutic applications. The V848E variant showed to be highly destabilizing in mCSM (Table 2) and results in a dramatic decrease in hydrophobicity (Figures 2A and 2B) even though it does not lead to any structural alteration. The Y1059 establishes two hydrogen bonds with residues R1032 and D1028 from the lateral chain. Upon change of a Tyrosine for a Cysteine occurs to the loss of the two hydrogen

bonds in the lateral chain (Figure 3A and 3B). In relation to the hydrophobicity, this variant results in a more hydrophobic pocket (Figures 3C and 3D).

The EGFR gene showed the four variants L858R, G779C, G779V and Y801C with maximum score 16. Although all variants were located at the TK domain and related to drugs, none of them led to alteration in structure. The 858-residue showed significant reduction in hydrophobicity upon alteration of a Leucine for an Arginine (Figure 4A and 4B). Regarding the Glycine alteration in the 779-residue (Figure 5A), it showed increased hydrophobicity upon change for and Cysteine (Figure 5B) and Valine (Figure 5C). In a similar way to variant Y1059C from VEGFR-2, the variant Y801C in EGFR also results in increased hydrophobicity upon the change of e Tyrosine for a Cysteine (Figure 6A and 6B).

Other 23 variants showed score 15 and ten of them were selected for further analysis due to the reports in therapeutic studies. Two variants were located at the extracellular domain (C329R and G428D) and eight at TK domain (G719S, G719D, G719C, K745R, G796S, H835L, A859V and V742A). Upon the alteration of a Cysteine on residue 329 for an Arginine it results in the establishment of one hydrogen bonds with the residue S315 (Figure 7A and 7B). The G428D variant, although did not change any hydrogen bond in the structure, led to an increase in hydrophily due to the change for a glutamic acid (Figure 8A and 8B), and was found highly destabilizing for the protein structure in mCSM evaluation.

In the TK domain, the variants V742A, A859V and G796S did not exert any effect in polar contacts established, both predicted as destabilizing the structure. Interestingly, while the V742A variant lead to decrease in hydrophobicity (Figure 9A and 9B), the A859V variant had the contrary effect (Figure 10A and 10B).

The G719 residue presents three different variants, all of which establish one hydrogen bond with the side chain of T725 (Figures 11A-D). The three variants showed contradictory evaluation in the structural stability algorithms and the assessment of hydrophobicity alteration showed that variant 719D resulted in increased hydrophily (Figure 12A and 12B) and variant 719C increased the hydrophobicity (Figure 12C).

The K745R variant leads to the loss of two hydrogen bond, due to the change of the Lysine involved in two polar contacts, with a contradictory evaluation in the stability prediction algorithm. K745 establish two hydrogen bond with E762 (Figure 13A) while the 745R variant loses the two bonds with E762 (Figure 13B). Regarding H835 residue, its replace by a Leucine lead to loss of one hydrogen bonds with T854 residue. It was also contradictory in the predictive results of the protein stability through the algorithms.

3. Discussion

Angiogenesis is one of the hallmarks of cancer because it allows tumors development and progression (Hanahan and Weinberg, 2011). Several therapeutic agents have come to place in order to inhibit angiogenesis both by development of monoclonal antibodies and through tyrosine-kinase inhibitors (TKIs). The tailor of the right therapy is pivotal to patient outcome once cancer cells acquire resistance through new mutations or activation of parallel pathways, overcoming the drug effects (Harris and McCormick, 2010). Therefore, the occurrence of non-synonymous SNVs in protein structure could potentially lead to impaired biologic activity.

For the VEGFR-2 only variants of the tyrosine-kinase domain reached higher scores. The selected V848E variant is a hydrophobic residue in the N-terminus of β2–sheet (Roskoski, 2017), found inactivating tyrosine-kinase activity (Dougher and

Terman, 1999) due to its interference K868 residue, located in β3−sheet, and responsible for ATP phosphates binding to VEGFR-2 (Roskoski, 2017). In addition, the residue Y1059 responsible for the auto-phosphorilation of the VEGFR-2 activation loop, necessary for tyrosine-kinase signaling (Dougher and Terman, 1999; Roskoski, 2017), showed Y1059C variant destabilizing the VEGFR-2 tyrosine-kinase domain structure. These findings provide a very important status for these mutations in targeted therapy, as both the Y1059C and V848E results in impaired tyrosine-kinase activity (Kendall et al., 1999; Roskoski, 2017) thus patients with this SNVs do not have the same benefits in TKI therapy as the patients without the variants.

Sorafenib targets VEGFR-2, and also VEGFR-3, RAF, RET, PDGR. It was already approved for treating advanced renal cell carcinoma, hepatocellular carcinoma and thyroid carcinoma. Sunitinib, as sorafenib, is a muti-target TKI able to inhibit VEGFR-2, as well VRGFR-3, PDGFR−β, CSF1R, RET, C-KIT and FGFR; approved for treating advanced renal cell carcinoma, gastrointestinal stromal tumors and metastatic pancreatic neuroendocrine tumors. Vandetanib, able to inhibit both VEGFR-2 and EGFR, was approved for the treatment of metastatic medullary thyroid cancer (Chu and Otterson, 2016).

Mutations and upregulation of EGFR can be found in around 30% of NSCLC, glioblastoma and anal cancer, which cetuximab and panitumumab can be used for treatment (Pandey and Mahadevan, 2014). Despite cetuximab binds to the extracellular portion of EGFR to block its dimerization, it is not effective against some EGFR-variants in NSCLC, like L858R that leads to EGFR activity independent of dimerization (Jia et al., 2016). This is one the most common mutations of EGFR conferring enhanced sensitivity of EGFR to TKIs (gefitinib and erlotinib) treatment through enlargement of the N-terminal portion of the activation loop (Harris and McCormick, 2010; Jia et al.,

2016). The same occurs for C329R variant in the cystein-rich domain 2, responsible for receptor dimerization of EGFR and identified in myeloproliferative neoplasm. This variant induces ligand-independent covalent receptor dimerization thus enhancing the EGFR signalling pathway (Casolari et al., 2017), maybe due to the increase in the number of hydrogen bonds. These variants are potential biomarkers for applying precise medicine and overcome cancer progression.

The G428D variant of EGFR leads to 100-fold weaker binding to EGF (Ganetzky et al., 2015) possibly due to the alteration in hydrophobicity, showing its importance assumed as deleterious in 15 different algorithms despite the contradictory results in predicting the stability effect. Nevertheless, none of the SNVs in the extracellular portion of EGFR where in the residues directly involved in EGFR inhibition through the use of mAbs.

In EGFR intracellular portion, variants in residue 719 were detected in 1% of highly-responsive patients to TKI inhibitors of EGFR (Shostak and Chariot, 2015).The G719S variant retains catalytical competence even after substitution of the conserved residue (Roskoski, 2014), showing less impact in EGFR signalling compared to other variants, as observed in our algorithm results. Studies found that L858R leads to an increased EGFR activity by 50-fold while G719S only increases in 10-fold. The inhibition of EGFR with gefitinib leads to a 97-fold and 6-fold effectiveness of inhibition (Roskoski, 2014). On the opposite direction, the V742A variant is associated with resistance to gefitinib in NSCLC treatments (Massarelli et al., 2013; Shih et al., 2006).

The K745 residue is responsible of binding to the β–phosphates of ATP to the α-C-helix, the conserved E762 occurs near the centre of the α-C-helix and the presence of a interaction between K745 and E762 is a prerequisite for the formation of the

EGFR's active state (Roskoski, 2014). K745R variant was found as potential deleterious, and *in vivo* studies found this SNV leading to the loss of tyrosine-kinase signalling (Brewer et al., 2010).

The G779C and G779V variants were identified in NSCLC patients (Chen et al., 2016; Heon et al., 2010), but no change in the number of hydrogen bonds or residue involved was found in our results. Not so far, the G796 residue is one of the conserved residues in a hinge (L792-H796) targeted by the ATP by a hydrogen bond. The G796S variant, predicted as one of the most deleterious in our analysis, can be very important for the effective treatment, once TKIs compete with ATP for binding to EGFR tyrosine-kinase domain, being able to establish hydrogen bonds with residues of the connecting hinge too (Roskoski, 2014). The adjacent residue C797 is responsible for establishing a covalent bond with afatinib molecule, an irreversible TKI that also binds to K745, H762 and M793 residues (Roskoski, 2014). Then, mutations in this hinge can reduce the effectiveness of the treatment.

In gastric cancer, the presence of Y801C variants has an unclear impact in TKI efficacy (Z. Liu et al., 2011), although it was found associated with resistance to TKI therapy (Massarelli et al., 2013). The severe destabilization prediction of this variant over the EGFR protein may be the cause, avoiding the binding of the TKI due to the removal of the aromatic ring of the Tyrosine and high change of hydrophobicity characteristic. The A859V variant is another variant associated with resistance to TKI treatment in NSCLC (Massarelli et al., 2013). In pulmonary neuroendocrine tumour A859V was found activating EGFR signalling in a similar way to L858R (Vollbrecht et al., 2015).

A total of 37 variants were found in the two highest algorithm scores in our analysis. However, 21 variants could not be evaluated due to the lack of information,

which three in VEGF (C266Y, V258M and R262Q); five in VEGFR-2 (L902Q, S1100F, R1051W, E828K and P1105L); and 13 variants in EGFR (L748R, C620Y, A822T, P265H, L90F, E84K, E884G, C231G, L448F, Y88C, Y285H, V902L and D247N). Although the computational approaches have been used for prospecting drug targets in metabolic pathways, with predictive methodologies that saves time and reduces the costs of the *in vitro* tests (Borba et al., 2017), it is important to confront the results in vivo. Then, even the importance of 16 variants has been described here, many others need to be studied *in vivo* to validate the prediction.

Conclusion

VEGFR-2 and EGFR are known as target for cancer treatment through monoclonal antibodies and tyrosine kinase inhibitors. In addition, they showed to be good candidates to predict the effectiveness of the therapy, helping in the access to the 'Precise Medicine Era'. However, further studies are necessary to determine the prevalence of the described SNV in different types of cancer and their role in drug effectiveness.

References

Akhtar, M.J., Ahamed, M., Alhadlaq, H. a., Alrokayan, S. a., Kumar, S., 2014. Targeted anticancer therapy: Overexpressed receptors and nanotechnology. Clin. Chim. Acta 436, 78–92. doi:10.1016/j.cca.2014.05.004

Bellone, S., Frera, G., Landolfi, G., Romani, C., Bandiera, E., Tognon, G., Roman, J.J., Burnett, A.F., Pecorelli, S., Santin, A.D., 2007. Overexpression of epidermal

growth factor type-1 receptor (EGF-R1) in cervical cancer: Implications for Cetuximab-mediated therapy in recurrent/metastatic disease. Gynecol. Oncol. 106, 513–520. doi:10.1016/j.ygyno.2007.04.028

Borba, M.A., Castelletti, C., Filho, J., Martins, D., 2017. Point-of-care devices : the next frontier in personalized chemotherapy. Futur. Sci.

Borba, M.A., Melo-Neto, R.P., Glauber M Leitão, C., HM Castelletti, J.L.L.-F.& D.B., Martins, 2016. Evaluating the impact of missenses mutations in CYP2D6*7 and CYP2D6*14A: does it compromise tamoxifen metabolism? Pharmacogenomics 17, 561–570. doi:10.2217/pgs-2015-0003

Brewer, M.R., Choi, S.H., Alvarado, D., Moravcevic, K., Lemmon, M. a, Carpenter, G., 2010. The Juxtamembrane Region of the EGFR functions as an activation domain. Mol. Cell 34, 641–651. doi:10.1016/j.molcel.2009.04.034.The

Calvetti, L., Pilotto, S., Carbognin, L., Ferrara, R., Caccese, M., Tortora, G., Bria, E., 2015. The coming of ramucirumab in the landscape of anti-angiogenic drugs: potential clinical and translational perspectives. Expert Opin. Biol. Ther. 15, 1359–1370. doi:10.1517/14712598.2015.1071350

Capdevila, J., Elez, E., Macarulla, T., Ramos, F.J., Ruiz-Echarri, M., Tabernero, J., 2009. Anti-epidermal growth factor receptor monoclonal antibodies in cancer treatment. Cancer Treat. Rev. 35, 354–63. doi:10.1016/j.ctrv.2009.02.001

Cardoso, M. de F.S., Castelletti, C.H.M., Lima-Filho, J.L. de, Martins, D.B.G., Teixeira, J.A.C., 2017. Putative biomarkers for cervical cancer: SNVs, methylation and expression profiles. Mutat. Res. Mutat. Res. 773, 161–173. doi:10.1016/j.mrrev.2017.06.002

Casolari, D.A., Nguyen, T., Butcher, C.M., Iarossi, D.G., Hahn, C.N., Bray, S.C.,

Neufing, P., Parker, W.T., Feng, J., Maung, K.Z.Y., Wee, A., Vidovic, L., Kok, C.H., Bardy, P.G., Branford, S., Lewis, I.D., Lane, S.W., Scott, H.S., Ross, D.M., D'Andrea, R.J., 2017. A novel, somatic, transforming mutation in the extracellular domain of Epidermal Growth Factor Receptor identified in myeloproliferative neoplasm. Sci. Rep. 7, 2467. doi:10.1038/s41598-017-02655-7

Chen, V.B., Arendall, W.B., Headd, J.J., Keedy, D.A., Immormino, R.M., Kapral, G.J., Murray, L.W., Richardson, J.S., Richardson, D.C., 2010. MolProbity: All-atom structure validation for macromolecular crystallography. Acta Crystallogr. Sect. D Biol. Crystallogr. 66, 12–21. doi:10.1107/S0907444909042073

Chen, Y., Ye, L., Stanford, R., Zhang, D., Zhang, X., Wei, W., 2016. Distinct epithelial growth factor receptor mutation profile in non-small‐cell lung cancer patients from the Xuanwei area of China. Mol. Clin. Oncol. 749–755. doi:10.3892/mco.2016.805

Chu, B.F., Otterson, G.A., 2016. Incorporation of Antiangiogenic Therapy Into the Non–Small-Cell Lung Cancer Paradigm. Clin. Lung Cancer 17, 493–506. doi:10.1016/j.cllc.2016.05.020

Clarke, J.M., Hurwitz, H.I., 2014. Targeted inhibition of VEGF Receptor-2: An update on Ramucirumab. Expert Opin. Biol. Ther. 13, 1187–1196. doi:10.1517/14712598.2013.810717.Targeted

Dassonville, O., Bozec, A., Fischel, J.L., Milano, G., 2007. EGFR targeting therapies: Monoclonal antibodies versus tyrosine kinase inhibitors. Similarities and differences. Crit. Rev. Oncol. Hematol. 62, 53–61. doi:10.1016/j.critrevonc.2006.12.008

Delcombel, R., Janssen, L., Vassy, R., Gammons, M., Haddad, O., Richard, B.,

Letourneur, D., Bates, D., Hendricks, C., Waltenberger, J., Starzec, A., Sounni, N.E., Noël, A., Deroanne, C., Lambert, C., Colige, A., 2013. New prospects in the roles of the C-terminal domains of VEGF-A and their cooperation for ligand binding, cellular signaling and vessels formation. Angiogenesis 16, 353–371. doi:10.1007/s10456-012-9320-y

Dougher, M., Terman, B.I., 1999. Autophosphorylation of KDR in the kinase domain is required for maximal VEGF-stimulated kinase activity and receptor internalization. Oncogene 18, 1619–1627. doi:10.1038/sj.onc.1202478

Ferlay, J., Soerjomataram, I., Ervik, M., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D., Forman, D., Bray, F., 2012. GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11 [WWW Document]. Lyon, Fr. Int. Agency Res. Cancer; 2013. URL http://globocan.iarc.fr (accessed 10.28.16).

Ferrara, N., Hillan, K.J., Gerber, H.-P., Novotny, W., 2004. Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. Nat. Rev. Drug Discov. 3, 391–400. doi:10.1038/nrd1381

Gadducci, A., Elena, M., Greco, C., 2013. Tissue biomarkers as prognostic variables of cervical cancer. Crit. Rev. Oncol. / Hematol. 86, 104–129. doi:10.1016/j.critrevonc.2012.09.003

Ganetzky, R., Finn, E., Bagchi, A., Zollo, O., Conlin, L., Deardorff, M., Harr, M., Simpson, M.A., Mcgrath, J.A., Zackai, E., Lemmon, M.A., Sondheimer, N., 2015. EGFR mutations cause a lethal syndrome of epithelial dysfunction with progeroid features. Mol. Genet. Genomic Med. 3, 452–458. doi:10.1002/mgg3.156

Gu, F., Li, X., Kong, J., Pan, B., Sun, M., Zheng, L., Yao, Y., 2013. VEGF111b, a new

member of VEGFxxxb isoforms and induced by mitomycin C, inhibits angiogenesis. Biochem. Biophys. Res. Commun. 441, 18–24. doi:10.1016/j.bbrc.2013.09.144

Hanahan, D., Weinberg, R.A., 2011. Hallmarks of cancer: the next generation. Cell 144, 646–674. doi:10.1016/j.cell.2011.02.013

Harris, T.J.R., McCormick, F., 2010. The molecular pathology of cancer. Nat. Rev. Clin. Oncol. 7, 251–65. doi:10.1038/nrclinonc.2010.41

Heon, S., Yeap, B.Y., Britt, G.J., Costa, D.B., Rabin, M.S., Jackman, D.M., Johnson, B.E., 2010. Development of central nervous system metastases in patients with advanced non-small cell lung cancer and somatic EGFR mutations treated with gefitinib or erlotinib. Clin. Cancer Res. 16, 5873–5882. doi:10.1158/1078-0432.CCR-10-1588

Hoeben, A.N.N., Landuyt, B., Highley, M.S.M., Wildiers, H., Oosterom, A.T.V.A.N., Bruijn, E.A.D.E., Van Oosterom, A.T., De Bruijn, E.A., 2004. Vascular endothelial growth factor and angiogenesis. Pharmacol. Rev. 56, 549–580. doi:10.1124/pr.56.4.3.549

Hofheinz, R.-D., Lorenzen, S., 2015. Ramucirumab as second-line treatment for patients with metastatic esophagogastric adenocarcinoma. Expert Rev. Anticancer Ther. 15, 607–14. doi:10.1586/14737140.2015.1052412

Jia, Y., Yun, C.-H., Park, E., Ercan, D., Manuia, M., Juarez, J., Xu, C., Rhee, K., Chen, T., Zhang, H., Palakurthi, S., Jang, J., Lelais, G., DiDonato, M., Bursulaya, B., Michellys, P.-Y., Epple, R., Marsilje, T.H., McNeill, M., Lu, W., Harris, J., Bender, S., Wong, K.-K., Jänne, P.A., Eck, M.J., 2016. Overcoming EGFR(T790M) and EGFR(C797S) resistance with mutant-selective allosteric

inhibitors. Nature 534, 129–132. doi:10.1038/nature17960

Kendall, R.L., Rutledge, R.Z., Mao, X., Tebben, A.J., Hungate, R.W., Thomas, K.A., 1999. Vascular endothelial growth factor receptor KDR tyrosine kinase activity is increased by autophosphorylation of two activation loop tyrosine residues. J. Biol. Chem. 274, 6453–60.

Laimer, J., Hofer, H., Fritz, M., Wegenkittl, S., Lackner, P., 2015. MAESTRO--multi agent stability prediction upon point mutations. BMC Bioinformatics 16, 116. doi:10.1186/s12859-015-0548-6

Lee, C.M., Lee, R.J., Hammond, E., Tsodikov, A., Dodson, M., Zempolich, K., Gaffney, D.K., 2004. Expression of HER2neu (c-erbB-2) and epidermal growth factor receptor in cervical cancer: Prognostic correlation with clinical characteristics, and comparison of manual and automated imaging analysis. Gynecol. Oncol. 93, 209–214. doi:10.1016/j.ygyno.2004.01.006

Levitzki, A., Klein, S., 2010. Signal transduction therapy of cancer. Mol. Aspects Med. 31, 287–329. doi:10.1016/j.mam.2010.04.001

Liu, X., Jian, X., Boerwinkle, E., 2011. dbNSFP: A lightweight database of human nonsynonymous SNPs and their functional predictions. Hum. Mutat. 32, 894–899. doi:10.1002/humu.21517

Liu, X., Wu, C., Li, C., Boerwinkle, E., 2016. dbNSFP v3.0: A One-Stop Database of Functional Predictions and Annotations for Human Nonsynonymous and Splice-Site SNVs. Hum. Mutat. 37, 235–241. doi:10.1002/humu.22932

Liu, Z., Liu, L., Li, M., Wang, Z., Feng, L., Zhang, Q., Cheng, S., Lu, S., 2011. Epidermal growth factor receptor mutation in gastric cancer. Pathology 43, 234–8. doi:10.1097/PAT.0b013e328344e61b

Maria, J., Prat, A., Gil-moreno, A., Pérez, J., Parera, M., 2008. Update on novel therapeutic agents for cervical cancer ☆ 110, 72–76. doi:10.1016/j.ygyno.2008.04.016

Massarelli, E., Johnson, F.M., Erickson, H.S., Wistuba, I.I., Papadimitrakopoulou, V., 2013. Uncommon Epidermal Growth Factor Receptor mutations in non-small cell lung cancer and their mechanisms of EGFR tyrosine kinase inhibitors sensitivity and resistance. Lung Cancer 80, 235–241. doi:10.1016/j.lungcan.2013.01.018

Nakai, K., Hung, M., Yamaguchi, H., 2016. Review Article A perspective on anti-EGFR therapies targeting triple-negative breast cancer 6, 1609–1623.

Pandey, M., Mahadevan, D., 2014. Monoclonal antibodies as therapeutics in human malignancies. Futur. Oncol. 10, 609–636. doi:10.2217/fon.13.197

Pires, D.E. V, Ascher, D.B., Blundell, T.L., 2014. MCSM: Predicting the effects of mutations in proteins using graph-based signatures. Bioinformatics 30, 335–342. doi:10.1093/bioinformatics/btt691

Roskoski, R., 2017. Vascular endothelial growth factor (VEGF) and VEGF receptor inhibitors in the treatment of renal cell carcinomas. Pharmacol. Res. 120, 116–132. doi:10.1016/j.phrs.2017.03.010

Roskoski, R., 2014. The ErbB/HER family of protein-tyrosine kinases and cancer. Pharmacol. Res. 79, 34–74. doi:10.1016/j.phrs.2013.11.002

Roskoski, R., 2008. VEGF receptor protein–tyrosine kinases: Structure and regulation. Biochem. Biophys. Res. Commun. 375, 287–291. doi:10.1016/j.bbrc.2008.07.121

Shih, J.-Y., Gow, C.-H., Yu, C.-J., Yang, C.-H., Chang, Y.-L., Tsai, M.-F., Hsu, Y.-C., Chen, K.-Y., Su, W.-P., Yang, P.-C., 2006. Epidermal growth factor receptor mutations in needle biopsy/aspiration samples predict response to gefitinib therapy

and survival of patients with advanced nonsmall cell lung cancer. Int. J. Cancer 118, 963–969. doi:10.1002/ijc.21458

Shostak, K., Chariot, A., 2015. EGFR and NF-??B: Partners in cancer. Trends Mol. Med. 21, 385–393. doi:10.1016/j.molmed.2015.04.001

Vollbrecht, C., Werner, R., Walter, R.F.H., Christoph, D.C., Heukamp, L.C., Peifer, M., Hirsch, B., Burbat, L., Mairinger, T., Schmid, K.W., Wohlschlaeger, J., Mairinger, F.D., 2015. Mutational analysis of pulmonary tumours with neuroendocrine features using targeted massive parallel sequencing: a comparison of a neglected tumour group. Br. J. Cancer 113, 1704–1711. doi:10.1038/bjc.2015.397

Wang, Y., Fei, D., Vanderlaan, M., Song, A., 2004. Biological activity of bevacizumab, a humanized anti-VEGF antibody in vitro. Angiogenesis 7, 335–345. doi:10.1007/s10456-004-8272-2

Table 1: Algorithms characteristics and cutoff used for evaluation

Algorithm	Method	Threshold (Score 1)	Observation
CADD	SVM	>=20	Variant in 1% of most deleterious
DANN	NN	>=0.96	Higher probability in damage
FATHMM-MKL	MKL	D	Analyses coding and non-coding variants
Fathmm-MKL	HMM	D	It is able to separate cancer promoting variants
LRT	LRT	LP	Compares conservative to neutral models.
M-CAP	SVM	D	Clinical pathogenicity classifier
MetaLR	LR	D	Higher likelihood of pathogenicity
MetaSVM	RK SVM	D	Higher likelihood of pathogenicity
Mutation Assessor	CEO	M/H	Do not uses Support Vector Machine
Mutation Taster	BC	D	The closer to 1, more reliable prediction
MutPred	RF	>0.75	High sensibility and specificity
Polyphen2 HDIV	NBSML	D	Highly used in SNVs impact research
PROVEAN	PB	D	The cut-off is provided in the algorithm
REVEL	RF	>0.375	High sensibility
SIFT	PB	D	Uses the homologous sequence

VEST3	RF	>0.95	The closer to 1, more likely deleterious

Legend: BC - Bayes classifier; CEO - Combinatorial entropy optimization; D – deleterious; H – High; HMM - Hidden Markov Models; LP – LRTpred; LRT - Likelihood ratio test; LR - Logistic regression; MKL - Multiple kernel learning; M – Medium; NB - Naïve Bayes; NN - Neural network; PB – PSI-BLAST; RF – random forest; RK - Radial kernel; SML – supervised machine-learning; SVM – support vector machine.

Table 2 – List of SNVs from EGFR and VEGFR-2 and their stability alterations upon SNVs.

SNV	MAESTRO	mCSM
VEGFR-2		
V848E	Destabilizing	Highly destabilizing
Y1059C	Destabilizing	Destabilizing
EGFR		
C329R	Destabilizing	Destabilizing
G428D	Stabilizing	Highly destabilizing
G719C	Stabilizing	Destabilizing
G719D	Stabilizing	Destabilizing
G719S	Stabilizing	Destabilizing
V742A	Destabilizing	Destabilizing
K745R	Stabilizing	Destabilizing
G779C	Destabilizing	Destabilizing
G779V	Destabilizing	Stabilizing
G796S	Destabilizing	Destabilizing
Y801C	Destabilizing	Highly destabilizing
H835L	Stabilizing	Destabilizing
L858R	Destabilizing	Destabilizing
A859V	Destabilizing	Destabilizing

Figure 1 – SNVs score distribution for VEGF (A), VEGFR2 (B) and EGFR (C).

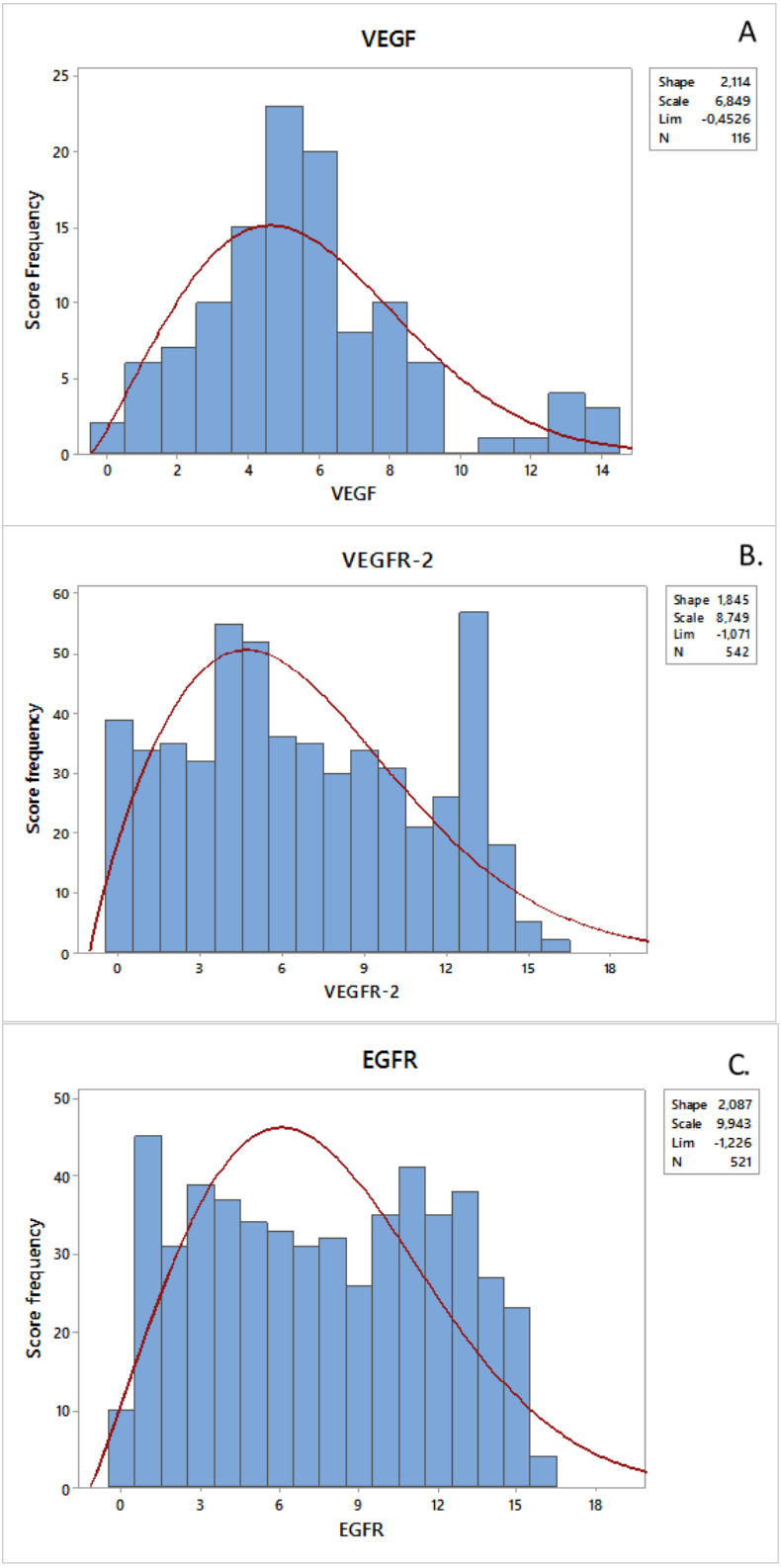


Figure 2 - Hydrophobicity change due to variant V848E in VEGFR2.

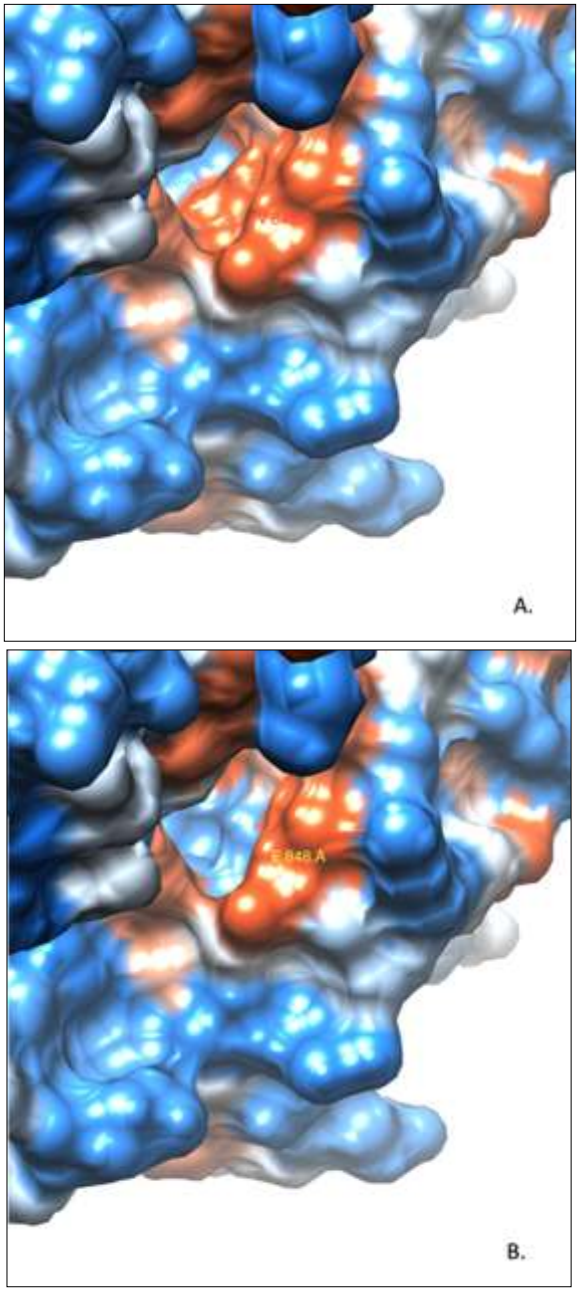


Figure 3 – Structural alteration on VEGFR2 due to the alteration of a Tyrosine (A) for a Cysteine (B) at the residue 1059 and the consequent hydrophobicity pattern alteration (C and D, for the wildtype and variant, respectively).

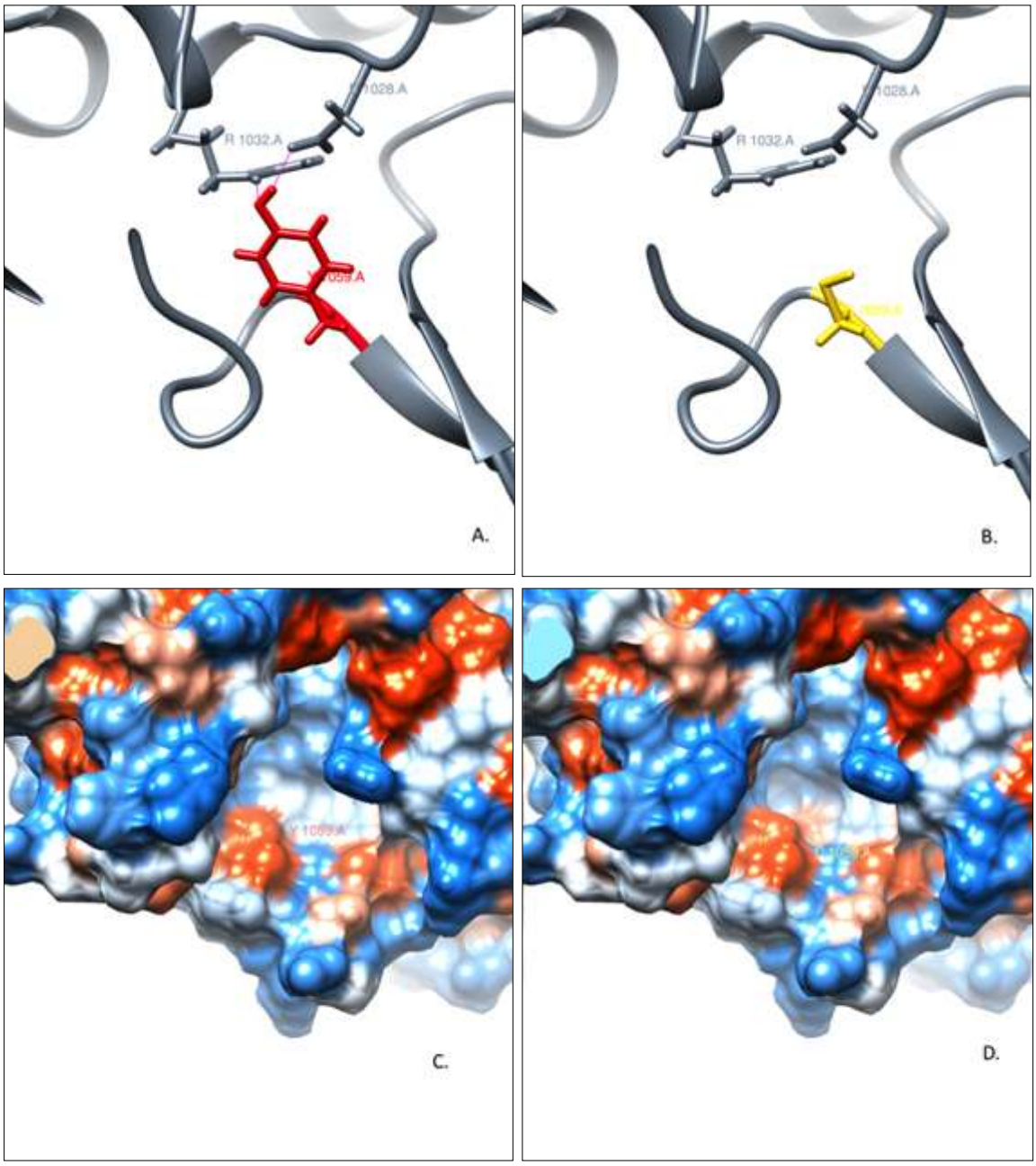


Figure 4 – Surface hydrophobicity alteration in the wildtype 858 residue of EGFR (A) and the variant residue (B)

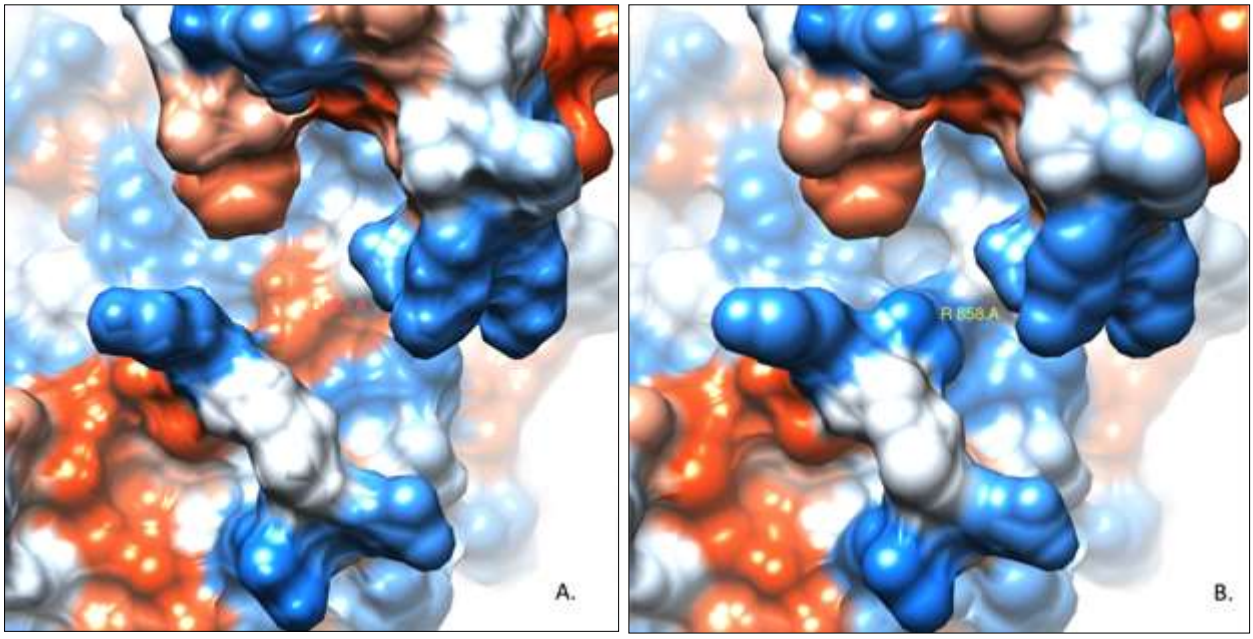


Figure 5 – Surface hydrophobicity alteration in the wildtype G779 residue of EGFR (A) and the variants Cysteine (B) and Valine (C) residues.

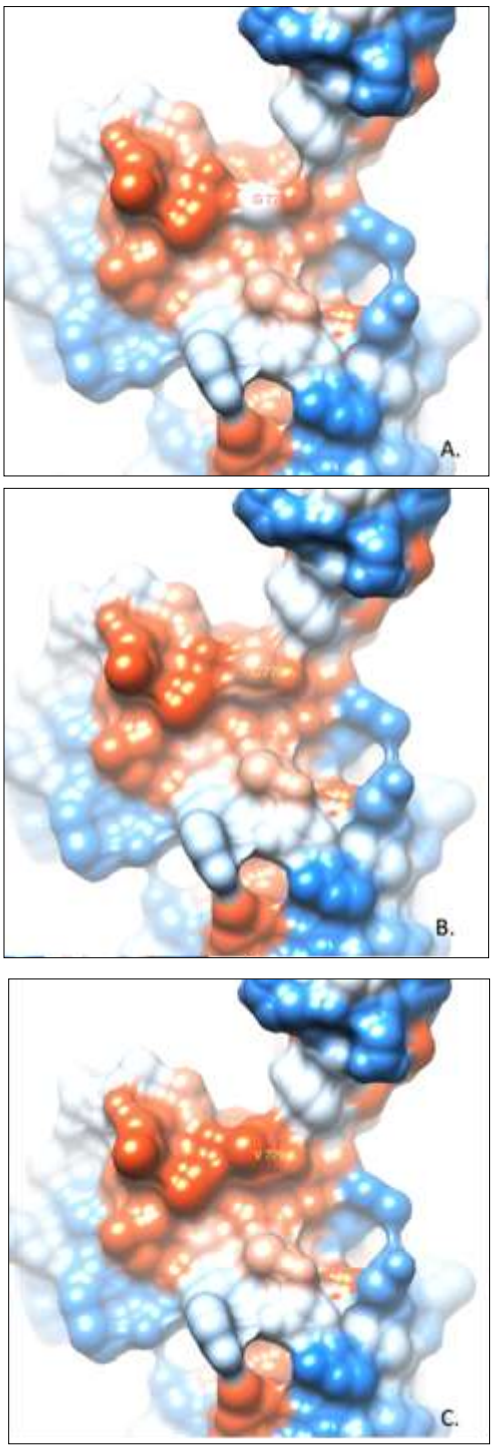


Figure 6 – Surface hydrophobicity alteration in the wildtype Y801 residue of EGFR (A) and the variant residue (B).

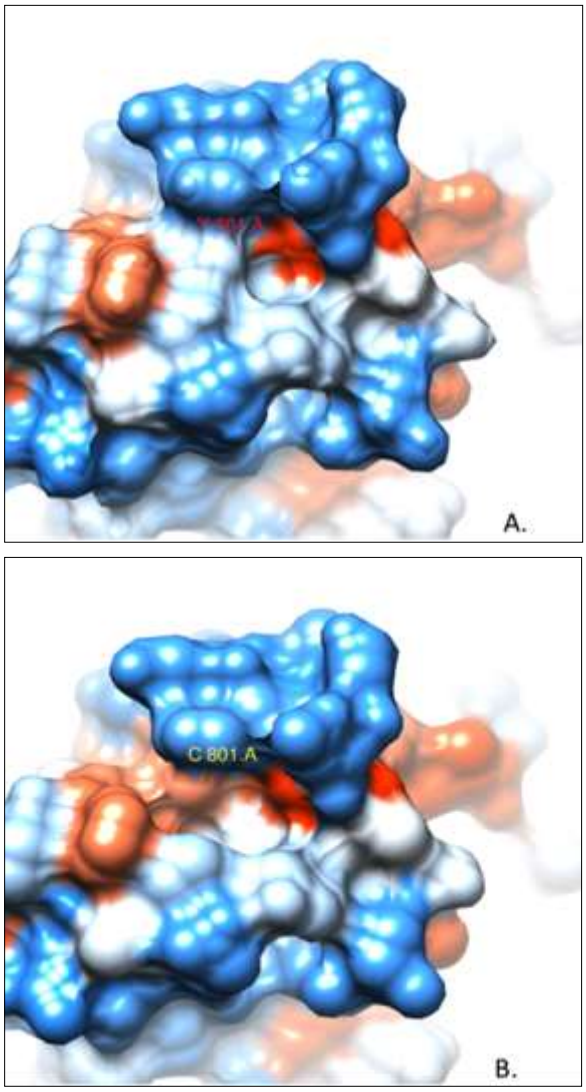


Figure 7 – Structural alteration of EGFR ectodomain upon alteration of C329 (A) for an Arginine (B).

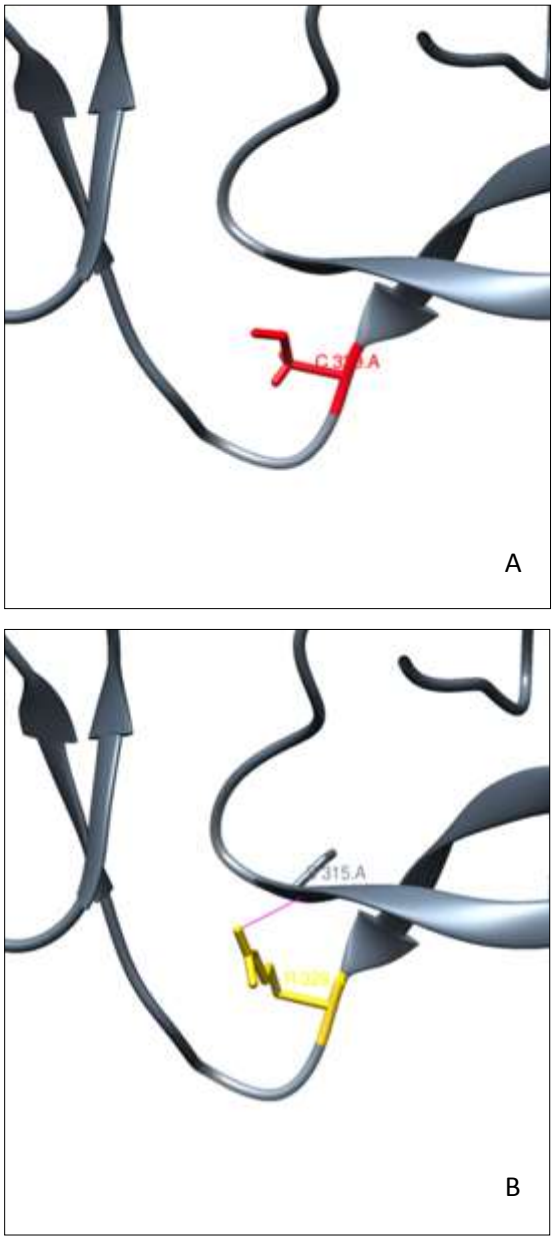


Figure 8 – Hydrophobicity alteration of the ectodomain residue 428 upon alteration of a Glycine (A) for a Glutamic Acid (B).

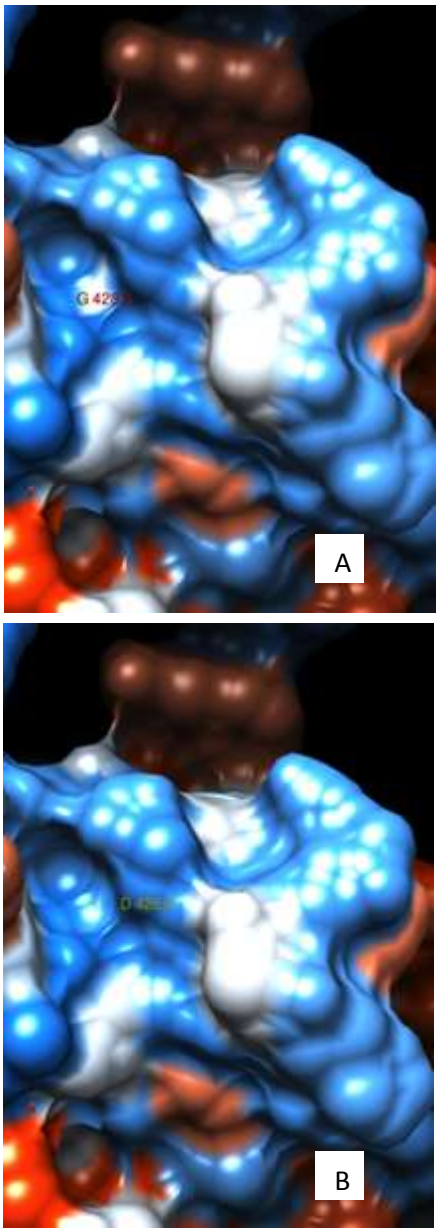


Figure 9 – Hydrophobicity alteration of the residue V742 (A) due to alteration for an Alanine (B)

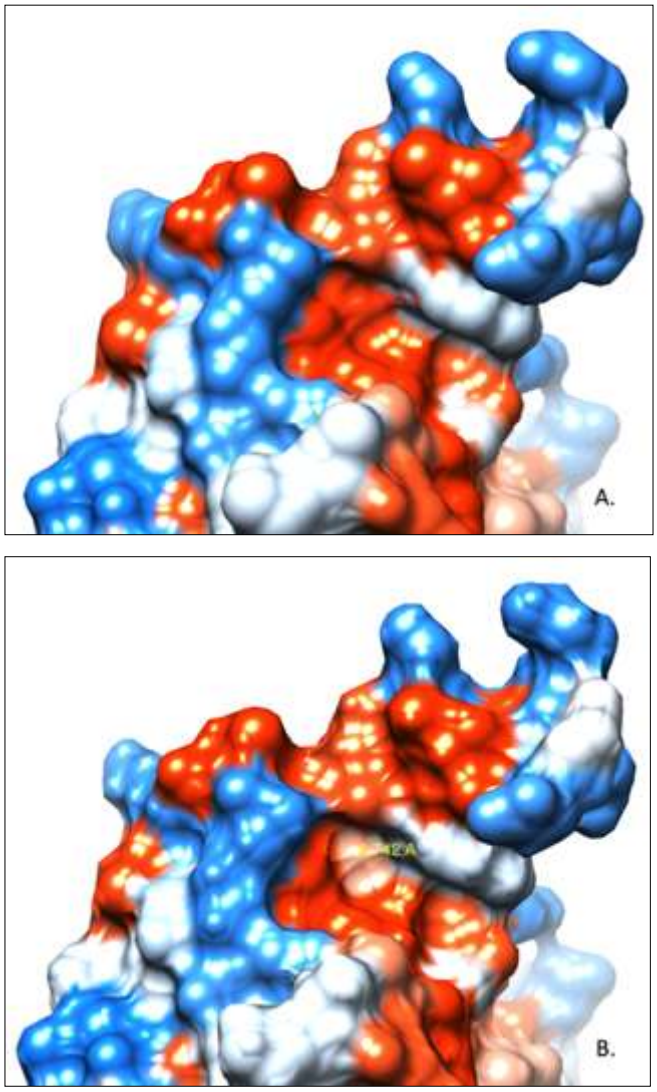


Figure 10 – Hydrophobicity alteration of the residue A859 (A) due to alteration for an Valine (B)

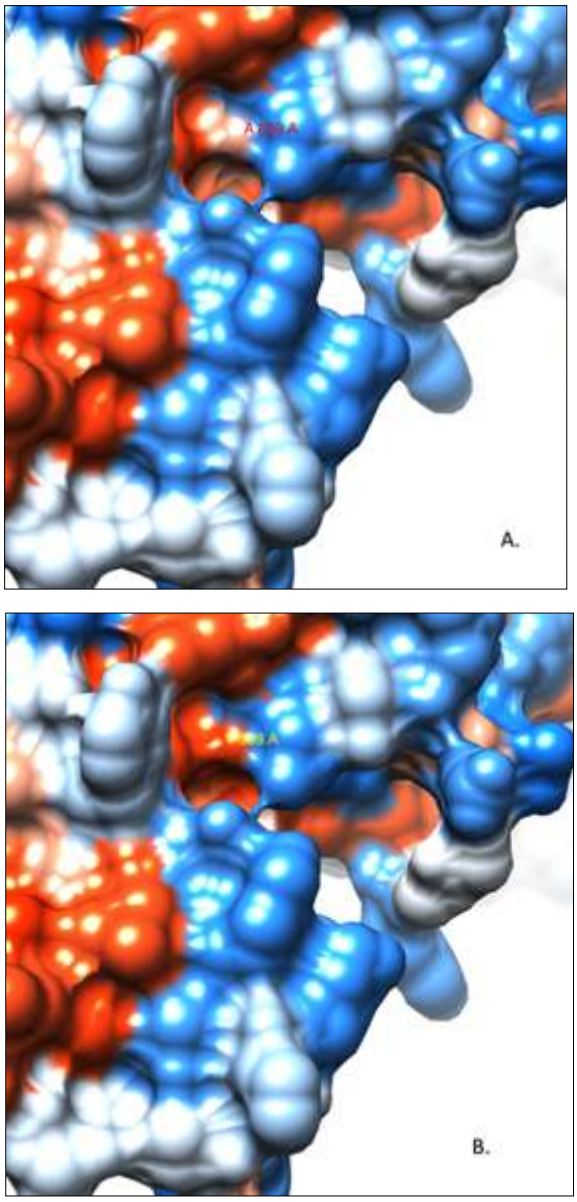


Figure 11 – Structural alteration of the residue G719 (A) for a Cysteine (B), Glutamic Acid (C) and Serine (D) from EGFR.

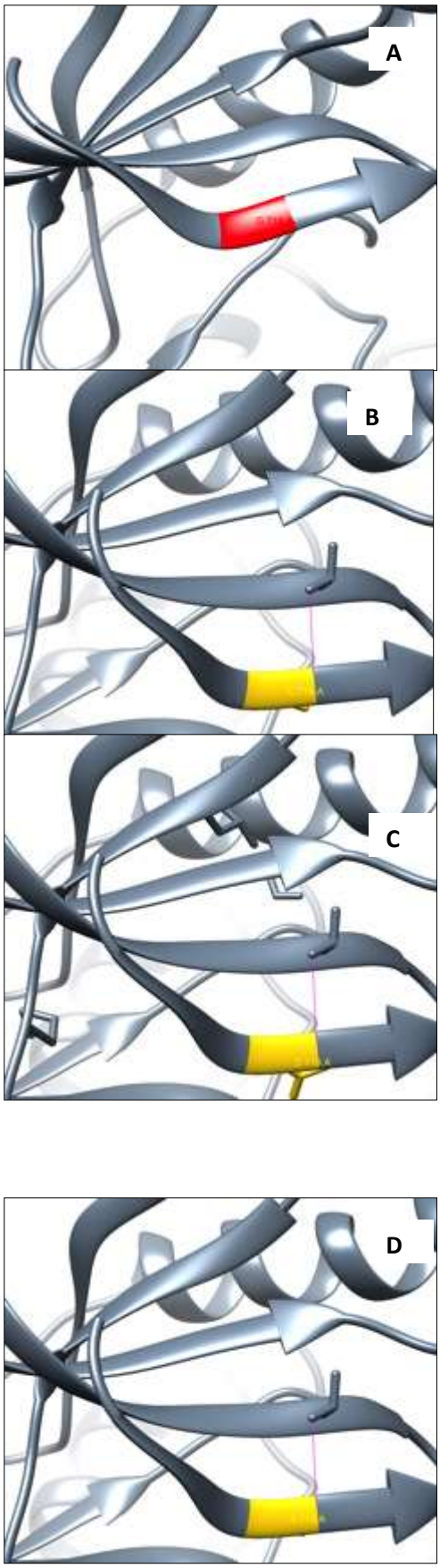


Figure 12 –Hydrophobicity alteration from the G719 (A) to a Glutamic Acid (B) and a Cysteine (C) from EGFR.

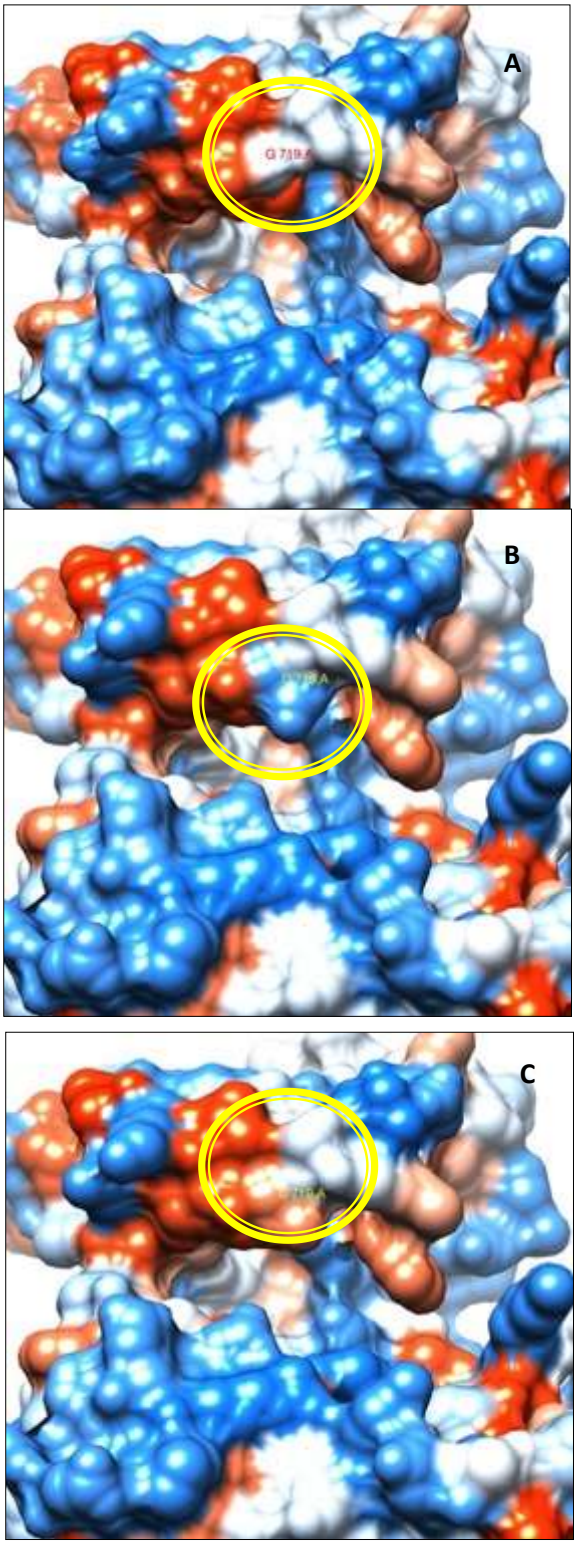
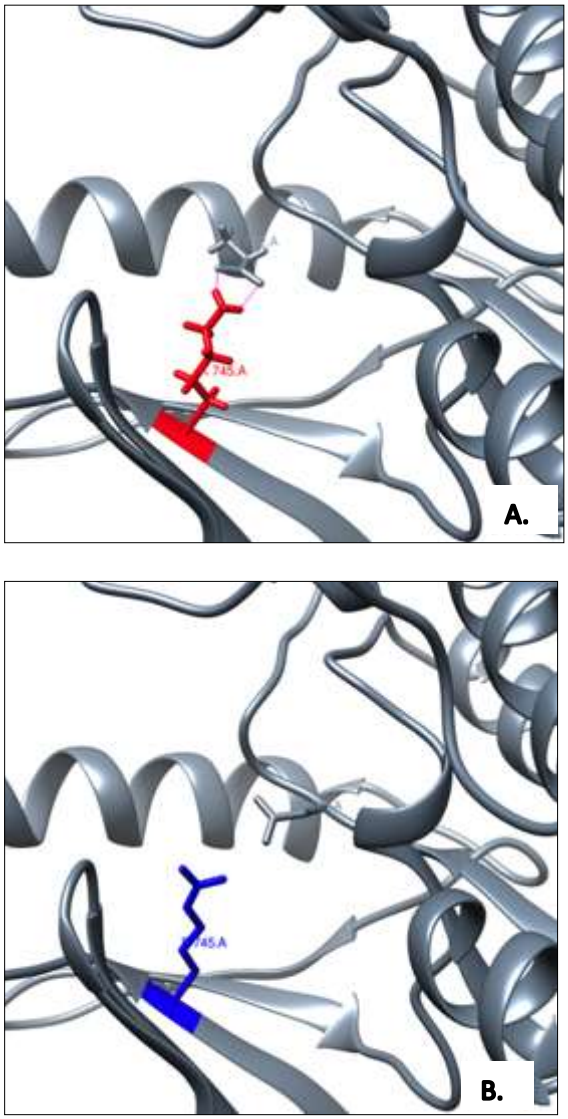


Figure 13 – Structural alteration of the residue K745 (A) upon alteration for an Arginine (B)



7 CONSIDERAÇÕES FINAIS

Neste trabalho, foi feita a análise dos níveis de expressão de Sirtuínas 1, 3, 5 e Interleucina 10 em pacientes acometidas por infecção por HPV e câncer cervical, verificando-se diminuição da expressão de IL-10 em câncer cervical, assim como aumento da expressão de SIRT5 nesta neoplasia.

Além disso, estabeleceu-se um grupo de 4 genes (CDH1, CDKN2A, RB1, TP53) como biomarcadores em câncer cervical estando afetados em três níveis moleculares: alteração na sequência de DNA, alteração do padrão de metilação e também alteração nos níveis de expressão destas moléculas. Outros dez genes foram identificados como sendo potenciais biomarcadores mas serão necessários mais estudos determinando a sua significância em pelo menos um destes aspectos moleculares. Adicionalmente, o uso de algoritmos de predição permitiu identificar 16 mutações com elevado potencial deletério, sendo 14 no EGFR e 02 no VEGFR-2 tendo impacto no direcionamento terapêutico de pacientes com estas mutações presentes.

Tais dados levaram à identificação de mutações que têm influência no direcionamento terapêutico em câncer, demonstrando o grande potencial das ferramentas de bioinformática não só neste campo mas também na determinação de biomarcadores para diagnóstico e progóstico em câncer cervical. Desta forma, este trabalho pretende contribuir para o desenvolvimento de painéis moleculares comerciais voltados para pacientes de câncer de cervical, assim como auxiliar no direcionamento terapêutico.

REFERÊNCIAS

A, N. S.; VALASALA, H.; KAMMA, S. In silico Evaluation of Nonsynonymous Single Nucleotide Polymorphisms in the ADIPOQ Gene Associated with Diabetes , Obesity , and Inflammation. **Avicenna J Med Biotech**, v. 7, n. 3, p. 121–127, 2015.

AKHTAR, M. J. et al. Targeted anticancer therapy: Overexpressed receptors and nanotechnology. **Clinica Chimica Acta**, v. 436, p. 78–92, 2014.

ANGIOLI, R. et al. Ten years of HPV vaccines: State of art and controversies. **Critical Reviews in Oncology/Hematology**, v. 102, p. 65–72, 2016.

APPLEBY, P. et al. Carcinoma of the cervix and tobacco smoking: Collaborative reanalysis of individual data on 13,541 women with carcinoma of the cervix and 23,017 women without carcinoma of the cervix from 23 epidemiological studies. **International Journal of Cancer**, v. 118, n. 6, p. 1481–1495, 2006.

BAVA, S. V; THULASIDASAN, A. K. T.; SREEKANTH, C. N. Cervical cancer: A comprehensive approach towards extermination. **Annals of Medicine**, v. 48, n. 3, p. 149–161, 2016.

BELLONE, S. et al. Overexpression of epidermal growth factor type-1 receptor (EGF-R1) in cervical cancer: Implications for Cetuximab-mediated therapy in recurrent/metastatic disease. **Gynecologic Oncology**, v. 106, p. 513–520, 2007.

BERRINGTON DE GONZÁLEZ, A.; GREEN, J. Comparison of risk factors for invasive squamous cell carcinoma and adenocarcinoma of the cervix: Collaborative reanalysis of individual data on 8,097 women with squamous cell carcinoma and 1,374 women with adenocarcinoma from 12 epidemiological studies. **International Journal of Cancer**, v. 120, n. 4, p. 885–891, 2007.

BORBA, M. A. et al. Evaluating the impact of missenses mutations in CYP2D6*7 and CYP2D6*14A: does it compromise tamoxifen metabolism? **Pharmacogenomics**, v. 17, n. 6, p. 561–570, 2016.

BOSHART, M. et al. A new type of papillomavirus DNA, its presence in genital cancer biopsies and in cell lines derived from cervical cancer. **The EMBO journal**, v. 3, n. 5, p. 1151–7, 1984.

CAO, J.; XIONG, L. Protein Sequence Classification with Improved Extreme Learning Machine Algorithms. v. 2014, 2014.

CAPDEVILA, J. et al. Anti-epidermal growth factor receptor monoclonal antibodies in cancer treatment. **Cancer treatment reviews**, v. 35, n. 4, p. 354–63, jun. 2009.

CASTLE, P. E. et al. The relationship of community biopsy-diagnosed cervical intraepithelial neoplasia grade 2 to the quality control pathology-reviewed diagnoses: An alts report. **American Journal of Clinical Pathology**, v. 127, n. 5, p. 805–815, 2007.

CHEN, K.; KURGAN, L. Handbook of Natural Computing. In: ROZENBERG, G.; BACK, T.; KOK, J. N. (Eds.). . **Handbook of Natural Computing**. [s.l: s.n.]. p. 585–622.

DELCOMBEL, R. et al. New prospects in the roles of the C-terminal domains of VEGF-A and their cooperation for ligand binding, cellular signaling and vessels formation. **Angiogenesis**, v. 16, n. 2, p. 353–371, 2013.

DING, Y. et al. Genetic polymorphisms and phenotypic analysis of drug-metabolizing enzyme CYP2C19 in a Li Chinese population. **International Journal of Clinical and Experimental Pathology**, v. 8, n. 10, p. 13201–13208, 2015.

FERLAY, J. et al. **GLOBOCAN 2012 v1.0, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 11**. Disponível em: <http://globocan.iarc.fr>. Acesso em: 28 out. 2016.

FERRARA, N. et al. Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. **Nature reviews. Drug discovery**, v. 3, n. 5, p. 391–400, 2004.

FROUSIOS, K. et al. Predicting the functional consequences of non-synonymous DNA sequence variants - evaluation of bioinformatics tools and development of a consensus strategy. **Genomics**, v. 102, n. 4, p. 223–228, 2013.

GADDUCCI, A.; ELENA, M.; GRECO, C. Tissue biomarkers as prognostic variables of cervical cancer. **Critical Reviews in Oncology / Hematology**, v. 86, n. 2, p. 104–129, 2013.

HERFS, M.; CHRISTOPHER, P. Cervical cancer: Squamocolumnar junction ablation—tying up loose ends? **Nature Publishing Group**, v. 12, n. 7, p. 378–380, 2015.

HOEBEN, A. N. N. et al. Vascular endothelial growth factor and angiogenesis. **Pharmacological reviews**, v. 56, n. 4, p. 549–580, 2004.

INSTITUTO NACIONAL DE CANCER JOSÉ ALENCAR GOMES DA SILVA. **INCA - Instituto Nacional de Câncer - Estimativa 2016**. [s.l: s.n.].

KANG, Y. J. et al. Optimal uptake rates for initial treatments for cervical cancer in concordance with guidelines in Australia and Canada: Results from two large cancer facilities. **Cancer Epidemiology**, v. 39, n. 4, p. 600–611, 2015.

ORCSMAROS, T.; SCHNEIDER, V.; DE, G. S. Next generation of network medicine: interdisciplinary signaling approaches. **Integrative Biology**, 2017.

KOVACIC, M. B. et al. Relationships of Human Papillomavirus Type, Qualitative Viral Load, and Age with Cytologic Abnormality. **Cancer Research**, v. 66, n. 20, p. 10112–10119, 2006.

LEE, C. M. et al. Expression of HER2neu (c-erbB-2) and epidermal growth factor receptor in cervical cancer: Prognostic correlation with clinical characteristics, and comparison of manual and automated imaging analysis. **Gynecologic Oncology**, v. 93, p. 209–214, 2004.

LEES, B. F.; ERICKSON, B. K.; HUH, W. K. Cervical cancer screening: Evidence behind the guidelines. **American Journal of Obstetrics and Gynecology**, v. 214, n. 4, p. 438–443, 2016.

LEVITZKI, A.; KLEIN, S. Signal transduction therapy of cancer. **Molecular Aspects of Medicine**, v. 31, n. 4, p. 287–329, 2010.

LI, B. et al. Automated inference of molecular mechanisms of disease from amino acid substitutions. **Bioinformatics**, v. 25, n. 21, p. 2744–2750, 2009.

LIU, M. et al. Comparison of random forest, support vector machine and back propagation neural network for electronic tongue data classification: Application to the recognition of orange beverage and Chinese vinegar. **Sensors and Actuators, B: Chemical**, v. 177, p. 970–980, 2013.

LIU, X. et al. dbNSFP v3.0: A One-Stop Database of Functional Predictions and Annotations for Human Nonsynonymous and Splice-Site SNVs. **Human Mutation**, v. 37, n. 3, p. 235–241, 2016.

LOPEZ MS, M. S. et al. Cervical cancer prevention and treatment in Latin America; Cervical cancer prevention and treatment in Latin America. **Journal of Surgical Oncology**, v. 9999, p. 1–4, 2017.

LORUSSO, D. et al. A systematic review comparing cisplatin and carboplatin plus paclitaxel-based chemotherapy for recurrent or metastatic cervical cancer. **Gynecologic Oncology**, v. 133, n. 1, p. 117–123, 2014.

LU, C. et al. Mechanisms for kinase-mediated dimerization of the epidermal growth factor receptor. **Journal of Biological Chemistry**, v. 287, n. 45, p. 38244–38253, 2012.

LUDWIG, J.; WEINSTEIN, J. Biomarkers in cancer staging, prognosis and treatment selection. **Nature Reviews Cancer**, v. 5, n. 11, p. 845–56, 2005.

MARIA, J. et al. Update on novel therapeutic agents for cervical cancer ☆. v. 110, p. 72–76, 2008.

MENDERES, G. et al. Immunotherapy and targeted therapy for cervical cancer: an update. **Expert review of anticancer therapy**, v. 7140, n. November 2015, 2015.

MOODY, C. A.; LAIMINS, L. A. Human papillomavirus oncoproteins: pathways to transformation. **Nature Reviews Cancer**, v. 10, p. 550–560, 2010.

NAGY, J. A.; DVORAK, A. M.; DVORAK, H. F. VEGF-A and the induction of pathological angiogenesis. **Annual review of pathology**, v. 2, p. 251–275, 2007.

NAKAI, K.; HUNG, M.; YAMAGUCHI, H. Review Article A perspective on anti-EGFR therapies targeting triple-negative breast cancer. v. 6, n. 8, p. 1609–1623, 2016.

PENG, L.; SONG, Z.; JIAO, S. Comparison of uncommon EGFR exon 21 L858R compound mutations with single mutation. **OncoTargets and Therapy**, v. 8, p. 905–910, 2015.

PLUMMER, M. et al. A 2-year prospective study of human papillomavirus persistence among women with a cytological diagnosis of atypical squamous cells of undetermined significance or low-grade squamous intraepithelial lesion. **The Journal of infectious diseases**, v. 195, n. 11, p. 1582–9, 2007.

RAGHAV, D.; SHARMA, V.; AGARWAL, S. M. Structural investigation of deleterious non-synonymous SNPs of EGFR gene. **Interdisciplinary Sciences: Computational Life Sciences**, v. 5, n. 1, p. 60–68, 2013.

RAJKUMAR, T. et al. Cervical carcinoma and reproductive factors: Collaborative reanalysis of individual data on 16,563 women with cervical carcinoma and 33,542 women without cervical carcinoma from 25 epidemiological studies. **International Journal of Cancer**, v. 119, n. 5, p. 1108–1124, 2006.

ROSKOSKI, R. Vascular endothelial growth factor (VEGF) signaling in tumor progression. **Critical Reviews in Oncology/Hematology**, v. 62, n. 3, p. 179–213, 2007.

ROSKOSKI, R. VEGF receptor protein–tyrosine kinases: Structure and regulation. **Biochemical and Biophysical Research Communications**, v. 375, n. 3, p. 287–291, out. 2008.

ROSKOSKI, R. The ErbB/HER family of protein-tyrosine kinases and cancer. **Pharmacological Research**, v. 79, p. 34–74, 2014.

SCHIFFMAN, M. et al. The carcinogenicity of human papillomavirus types reflects viral evolution. **Virology**, v. 337, n. 1, p. 76–84, 2005.

SCHIFFMAN, M. et al. Human papillomavirus and cervical cancer. **Lancet**, v. 370, n. 9590, p. 890–907, 2007.

SHINKARUK, S. et al. Vascular endothelial cell growth factor (VEGF), an emerging target for cancer chemotherapy. **Current Medicinal Chemistry. Anti-Cancer Agents**, v. 3, n. 2, p. 95–117, 2003.

SMITH, J. S. et al. Cervical cancer and use of hormonal contraceptives: A systematic review. **Lancet**, v. 361, n. 9364, p. 1159–1167, 2003.

STANLEY, M. Immune responses to human papillomavirus. **Vaccine**, v. 24, n. SUPPL. 1, p. 16–22, 2006.

STRICKLER, H. D. et al. Natural history and possible reactivation of human papillomavirus in human immunodeficiency virus-positive women. **Journal of the National Cancer Institute**, v. 97, n. 8, p. 577–586, 2005.

STRIMBU, K.; TAVEL, J. A. What are Biomarkers? **Curr Opin HIV AIDS**, v. 5, n. 6, p. 463–466, 2011.

SUKTITIPAT, B. et al. Molecular investigation by whole exome sequencing revealed a high proportion of pathogenic variants among Thai victims of sudden unexpected death syndrome. **Plos One**, v. 12, n. 7, p. e0180056, 2017.

VON KNEBEL DOEBERITZ, M. New markers for cervical dysplasia to visualise the genomic chaos created by aberrant oncogenic papillomavirus infections. **European Journal of Cancer**, v. 38, n. 17, p. 2229–2242, 2002.

WARD, C. W. et al. The insulin and EGF receptor structures: new insights into ligand-induced receptor activation. **Trends in Biochemical Sciences**, v. 32, n. 3, p. 129–137, 2007.

WHEELER, C. M. Advances in primary and secondary interventions for cervical cancer: human papillomavirus prophylactic vaccines and testing. **Nature clinical practice. Oncology**, v. 4, n. 4, p. 224–35, 2007.

WINER, R. L. et al. Genital human papillomavirus infection: Incidence and risk factors in a cohort of female university students. **American Journal of Epidemiology**, v. 157, n. 3, p. 218–226, 2003.

WOODMAN, C. . et al. The natural history of cervical HPV infection: unresolved issues - ProQuest. **Nature Reviews Cancer**, v. 7, n. January, p. 11–22, 2007.

WORKOWSKI, K. A.; BOLAN, G. A. **Sexually transmitted diseases treatment guidelines, 2015**. [s.l: s.n.]. v. 64

ZUR HAUSEN, H. Papillomaviruses and cancer: from basic studies to clinical application. **Nature reviews.Cancer**, v. 2, n. 5, p. 342–350, 2002.

ANEXO A - PATENTE

< Uso exclusivo do INPI >

INPI INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL
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INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL
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DIRPA	Tipo de Documento: Formulário	DIRPA	Página: 1/3
Título do Documento: Depósito de Pedido de Patente		Código: FQ001	Versão: 2
		Procedimento: DIRPA-PQ006	

Ao Instituto Nacional da Propriedade Industrial:
O requerente solicita a concessão de um privilégio na natureza e nas condições abaixo indicadas:

1. Depositante (71):

1.1 Nome: José Luiz de Lima Filho

1.2 Qualificação: Médico

1.3 CNPJ/CPF: 21638241449

1.4 Endereço Completo: Av. Prof. Morais Rego, s/n

1.5 CEP: 50670-901

1.6 Telefone: 81 2126 8484 1.7 Fax: 81 2126 8485

1.8 E-mail: joseluiz60@gmail.com

☐ continua em folha anexa

2. Natureza: ☒ Invenção ☐ Modelo de Utilidade ☐ Certificado de Adição

3. Título da Invenção ou Modelo de Utilidade (54):

PAINEL GENÉTICO NA PREDIÇÃO DE CÂNCER GINECOLÓGICO ASSOCIADO À SÍNDROME METABÓLICA

☐ continua em folha anexa

4. Pedido de Divisão: do pedido Nº **Data de Depósito:**

5. Prioridade: ☐ Interna (66) ☐ Unionista (30)

O depositante reivindica a(s) seguinte(s):

País ou Organização do depósito	Número do depósito (se disponível)	Data de depósito

☐ continua em folha anexa

6. Inventor (72):

☐ Assinale aqui se o(s) mesmo(s) requer(em) a não divulgação de seus nome(s), neste caso não preencher os campos abaixo.

6.1 Nome: José Luiz de Lima Filho

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6.3 CPF: 21638241449

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☒ continua em folha anexa

7. Declaração de divulgação anterior não prejudicial. ☐

Artigo 12 da LPI – período de graça.
Informe no item 11.13 os documentos anexados, se houver.

8. Declaração na forma do item 3.2 da Instrução Normativa PR nº 17/2013:

☐ Declaro que os dados fornecidos no presente formulário são idênticos ao da certidão de depósito ou documento equivalente do pedido cuja prioridade está sendo reivindicada.

9. Procurador (74):

9.1 Nome:

9.2 CNPJ/CPF: 9.3 API/OAB:

9.4 Endereço Completo:

9.5 CEP:

9.6 Telefone: 9.7 FAX:

9.8 E-mail:

☐ continua em folha anexa

10. Listagem de sequências biológicas.

Informe nos itens 11.9 ao 11.12 os documentos anexados, se houver. ☐

INSTITUTO NACIONAL DA PROPRIEDADE INDUSTRIAL
Sistema de Gestão da Qualidade
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Título do Documento: Depósito de Pedido de Patente		Código: FQ001	Versão: 2
		Procedimento: DIRPA-PQ006	

11. Documentos Anexados:

(Assinale e indique também o número de folhas):
(Deverá ser indicado o número total de somente uma das vias de cada documento).

		Documentos Anexados	folhas
☒	11.1	Guia de Recolhimento da União (GRU).	1
☐	11.2	Procuração.	
☐	11.3	Documentos de Prioridade.	
☐	11.4	Documento de contrato de trabalho.	
☒	11.5	Relatório descritivo.	6
☒	11.6	Reivindicações.	2
☒	11.7	Desenho(s) (se houver). Sugestão de figura a ser publicada com o resumo: nº, _____ por melhor representar a invenção (sujeito à avaliação do INPI).	1
☒	11.8	Resumo.	1
☐	11.9	Listagem de sequências em arquivo eletrônico: _____ nº de CDs ou DVDs (original e cópia).	
☐	11.10	Código de controle alfanumérico no formato de código de barras referente às listagem de sequências.	
☐	11.11	Listagem de sequências em formato impresso.	
☐	11.12	Declaração relativa à Listagem de sequências.	
☐	11.13	Outros (especificar) Lista de inventores	1

12. Total de folhas anexadas: 12 **fls.**

13. Declaro, sob as penas da Lei que todas as informações acima prestadas são completas e verdadeiras.

Recife, 21 de outubro de 2015

Local e Data

Assinatura e Carimbo

Prof. José Luiz de Lima Filho
Diretor
LIKA / UFPE
SIAPE-1133637

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PAINEL GENÉTICO NA PREDIÇÃO DE CÂNCER GINECOLÓGICO ASSOCIADO À SÍNDROME METABÓLICA

RELATÓRIO DESCRITIVO

Campo da Invenção

[01] A presente invenção refere-se ao campo de métodos e dispositivos baseados em um painel genético de biomarcadores para a identificação de predisposição ao desenvolvimento de neoplasias específicas da mulher (mama, ovário, endométrio e colo uterino) associadas à síndrome metabólica em pacientes recém-nascidas com crescimento fetal anormal, não excluindo outras doenças.

Antecedentes da Invenção

[02] O câncer é a segunda maior causa de morte no mundo, ultrapassado apenas pelas doenças cardiovasculares. Caracterizada por alterações morfológicas e funcionais a nível celular leva a proliferação anômala e multiplicação desordenada com capacidade invasiva para tecidos adjacentes. Existem mais de 200 tipos de cânceres, com incidência global desigual, e dependente de diversos fatores tais como idade, sexo, ambientais e comportamentais. Segundo a Organização Mundial de Saúde (OMS), estima-se que em 2030 serão cerca de 27 milhões de novos casos de câncer, com 17 milhões de mortes no mundo.

[03] Diversos tipos de cânceres são exclusivos das mulheres: colo uterino, endométrio e ovário, por exemplo. O câncer de mama, apesar de atingir ambos os sexos, apresenta maior prevalência em mulheres. Segundo o Instituto Nacional de Câncer (INCA), durante o biênio 2014/2015, estão previstos mais de 273.000 casos de câncer em mulheres, dos quais 84.290 (~30,8%) correspondem aos quatro tipos de neoplasias supracitados.

[04] A realização de diagnóstico precoce de câncer é crucial para melhor prognóstico e sobrevida das pacientes. No câncer do colo uterino, o método

padrão de diagnóstico é o exame citológico das células cervicais (através do Papanicolau), permitindo a avaliação da existência de alterações morfológicas. Para o câncer de mama recomenda-se a mamografia em mulheres com idade superior a 40 anos; ou inferior, no caso de apresentar fatores de risco genéticos e/ou familiares. Atualmente não existe nenhum tipo de rastreamento especifico para câncer endometrial e ovariano, a não ser que a paciente apresente sintomatologia e/ou fatores de risco hereditários.

[05] Existem inúmeros fatores de risco que podem culminar no desenvolvimento de câncer na mulher, dentre eles a obesidade (índice de massa corpórea – IMC > 30), considerado pelos órgãos de saúde como um grave problema de saúde pública com elevado impacto socioeconômico, estando associada à susceptibilidade de diabetes tipo 2, doenças cardiovasculares, síndrome metabólica, dentre outros. A morbidade e mortalidade relacionadas ao câncer aumentam com a presença de obesidade; contudo, os principais mecanismos de ação, sejam eles hormonais, inflamatórios ou metabólicos, não estão totalmente esclarecidos.

[06] Síndrome metabólica é um conjunto de patologias que aumentam o risco do desenvolvimento de doenças cardiovasculares, impactando a vida de milhões de pessoas. Embora a patogênese da Síndrome Metabólica não esteja completamente compreendida, está fortemente relacionada a estilos de vida caracterizados pela inatividade física e intensa oferta de alimentos gordurosos. Além dos estilos de vida, indivíduos com crescimento fetal alterado apresentam maior incidência ao risco de câncer quando associado a complexas variações genéticas e ambientais.

[07] Crescimento e desenvolvimento fetal são processos dinâmicos que necessitam de interações síncronas entre a mãe, placenta e feto, fundamentais ao recém-nascido (RN) na garantia de condições de sobrevivência e crescimento adequados. A desregulação homeostática intrauterina durante o desenvolvimento fetal pode influenciar a saúde do RN ao longo de sua vida, causando o *imprinting uterino*. Essa hipótese, conhecida como Hipótese de Barker (David Barker, 1995), sugere que trocas metabólicas *in utero* estabelecem padrões fisiológicos e estruturais que programam a saúde durante a vida adulta, principalmente em RN com alteração do crescimento fetal (pequenos para idade gestacional – PIG, e gigantes para idade gestacional –

GIG). Recém-nascidos PIG e GIG tendem a apresentar maior susceptibilidade a obesidade, hipertensão arterial e síndrome metabólica.

[08] A análise precoce de genes envolvidos na alteração do crescimento fetal, que favoreçam o desenvolvimento de câncer ginecológico em pacientes com síndrome metabólica é de extrema relevância. Neste sentido a busca por marcadores moleculares funcionais envolvidos na patogênese do câncer ginecológico trás as pacientes a possibilidade de um acompanhamento clínico preventivo dos fatores de risco.

[09] A patente **WO 1999015704 A1**, de 1 de abril de 1999, intitulada "*Genetic panel assay for susceptibility mutations in breast and ovarian cancer*", refere-se a uma invenção que pesquisa a predisposição de genes para o desenvolvimento de câncer de mama e ovário, focando especificamente os genes clássicos *BRCA1* e *BRCA2*. Além disso, também se refere a métodos e moléculas para a detecção da presença dessas mutações e polimorfismos. No entanto, os alvos moleculares dessa patente não focam na predição de câncer de colo de útero e endometrial, bem como não associa essas patologias à síndrome metabólica.

[10] Já a patente **US 20130338027 A1**, de 19 de dezembro de 2013, intitulada "*Predictive Markers For Cancer and Metabolic Syndrome*", apresentou como proposta a identificação de biomarcadores preditivos e respectivos métodos de utilização para a determinação da síndrome metabólica, em adição à doença cardiovascular associado com a obesidade, para o desenvolvimento de câncer de próstata. Focou-se na investigação do gene *FASN* (*fatty acid synthase*), sua conexão com expressão tumoral e IMC elevado, associando-o a outros genes (como *BDNF*) que apresentam risco elevado à síndrome metabólica, bem como agressividade do câncer prostático. Apesar de realizar um painel genético para predição de câncer e síndrome metabólica, foram investigados apenas alvos moleculares de interesse ao câncer de próstata.

[11] A patente **US 20140018259 A1** ("*Novel tumor marker determination"*), de 16 de janeiro de 2014, apresenta uma invenção que visa métodos para determinação de biomarcadores para tumores sólidos, com ênfase no câncer ginecológico (mama, cervical, endometrial e colo de útero). Através da seleção de diferentes marcadores tumorais, é possível monitorar a terapia do câncer, realizando melhor diagnóstico e prognóstico. Novamente,

trata-se de patente relacionada ao diagnóstico de neoplasias sem relação à síndrome metabólica, além de não permitir a análise preditiva dessas patologias.

[12] Já a patente **US 8961982 B2**, intitulada “Modulation of developmental immune programming and protection against cardiovascular diseases, diabetes, infectious diseases, and cancer”, de 24 de fevereiro de 2015, prevê um método de imunização contra influências *in utero* que podem contribuir ao desenvolvimento de diversas patologias comum a vida adulta, como doenças cardiovasculares, resistência à insulina, diabetes e câncer. No entanto, foca de forma generalista os cânceres, não apresentando proposta de imunização preventiva para câncer ginecológico.

[13] Nenhuma das patentes citadas acima possui semelhança com o método proposto de um painel genético para o diagnóstico preditivo da susceptibilidade das RNs ao desenvolvimento do câncer ginecológico em associação à síndrome metabólica, principalmente para aquelas que apresentaram alteração do crescimento fetal (PIGs e GIGs).

[14] Desta forma, o objetivo da presente invenção é disponibilizar a identificação e predição dos genes relacionados à probabilidade do desenvolvimento de câncer de colo de útero, mama, ovário e endometrial, associado à obesidade e síndrome metabólica, sendo uma forma de diagnóstico precoce ao risco de desenvolver estas patologias ainda no início da vida da paciente.

Descrição da Invenção

[15] A presente invenção descreve o método baseado em painel genético para a identificação de marcadores moleculares relacionados à predição do desenvolvimento de câncer de mama, colo uterino, ovário e endométrio, com ênfase em pacientes que apresentaram alteração do crescimento fetal e, por consequência, predisposição às doenças metabólicas na vida adulta. Não exclusivo a pacientes com alterações no crescimento fetal, o método pode ser aplicado a pacientes com crescimento dentro da normalidade, pacientes suscetíveis e com histórico familiar, e outros casos que se faça pertinente.

[16] O método visa à predição da susceptibilidade em RNs, com alteração no crescimento, ao desenvolvimento de síndrome metabólica, obesidade, resistência à insulina e dislipidemias, e sua correlação ao risco de câncer ginecológico. A presente invenção baseia-se em análises de dados, genômicos, metabolômicos, interactômicos e da literatura para a análise destas interações gênicas. A figura 1 descreve as etapas do processo laboratorial para diagnóstico molecular das pacientes.

[17] Para a presente invenção foram utilizados os dados experimentais disponíveis no MetaCore (Thomson Reuters, USA) relativos a potenciais biomarcadores a cada uma das patologias. Em câncer de mama, foram encontrados 7.730 biomarcadores; 3.000 para câncer de colo uterino; 10.272 para neoplasias associadas a endométrio e 2.631 para câncer de ovário. Efetuando a busca para síndrome metabólica verificou-se a existência de 1.145 potenciais biomarcadores. Após sobrepor toda a informação recolhida chegou-se à conclusão de que existem 11 genes comuns aos quatro tipos de câncer abrangidos nesta invenção e associados à presença de síndrome metabólica, sendo potenciais biomarcadores para a predição dessas doenças.

[18] O presente painel genético é constituído pelos genes: *ACE*, *CAV1*, *CLOCK*, *CXCL12*, *CYP11B2*, *DRD2*, *ADIPOR1*, *BDNF*, *CCL2*, *DPP4* e *ESR1* (Tabela 1). Esses genes e seus produtos serão usados como biomarcadores de susceptibilidade ao desenvolvimento de cânceres ginecológicos associados à síndrome metabólica em pacientes RNs com alteração do crescimento fetal.

[19] O painel desenvolvido visa determinar os níveis de expressão gênica, alterações epigenéticas, e polimorfismos e/ou mutações que possam interferir na susceptibilidade do desenvolvimento de cânceres ginecológicos associados à síndrome metabólica em pacientes RNs com crescimento intrauterino alterado.

Tabela 1

ACE	Angiotensin I Converting Enzyme
CAV1	Caveolin 1, Caveolae Protein, 22kDa
CLOCK	Clock Circadian Regulator
CXCL12	Chemokine (C-X-C Motif) Ligand 12
CYP11B2	Cytochrome P450, Family 11, Subfamily B, Polypeptide 2
DRD2	Dopamine Receptor D2
ADIPOR1	Adiponectin receptor 1
BDNF	Brain-derived neurotrophic factor
CCL2	Chemokine (C-C Motif) Ligand 2
DPP4	Dipeptidyl-Peptidase 4
ESR1	Estrogen receptor 1

REIVINDICAÇÕES

1. Painel genético na predição de câncer ginecológico associado à síndrome metabólica, caracterizado por prever precocemente o desenvolvimento das neoplasias ginecológicas, não excluindo outras doenças.

2. Painel genético caracterizado por prever no que diz respeito ao câncer ginecológico, a susceptibilidade ao câncer de mama, conforme reivindicação 1.

3. Painel genético caracterizado por prever no que diz respeito ao câncer ginecológico, a susceptibilidade ao câncer de colo de útero, conforme reivindicação 1.

4. Painel genético caracterizado por prever no que diz respeito ao câncer ginecológico, a susceptibilidade ao câncer endometrial, conforme reivindicação 1.

5. Painel genético caracterizado por prever no que diz respeito ao câncer ginecológico, a susceptibilidade ao câncer de ovário, conforme reivindicação 1.

6. Painel genético caracterizado pela predição de câncer ginecológico em pacientes que apresentam síndrome metabólica

7. Painel genético na predição de câncer ginecológico associado à síndrome metabólica, caracterizado por um método de detecção em amostras, no que diz respeito à possibilidade de desenvolvimento de câncer ginecológico associado à resistência à insulina, conforme a reivindicação 6.

8. Painel genético na predição de câncer ginecológico associado à síndrome metabólica, caracterizado por um método de detecção em amostras, no que diz respeito à possibilidade de desenvolvimento de câncer ginecológico associado à obesidade, conforme a reivindicação 6.

9. Painel genético na predição de câncer ginecológico associado à síndrome metabólica, caracterizado pela detecção de um ou mais biomarcadores selecionados em um grupo consistindo de *ACE*, *CAV1*, *CLOCK*, *CXCL12*, *CYP11B2*, *DRD2*, *ADIPOR1*, *BDNF*, *CCL2*, *DPP4* e *ESR1* em que a alteração de um ou mais dos biomarcadores, em comparação com um padrão, indicará uma susceptibilidade ao desenvolvimento do fenótipo patológico.

10. Painel genético caracterizado pela detecção dos padrões dos níveis de expressão dos potenciais biomarcadores descritos no método de reivindicação 9.

11. Painel genético caracterizado pela detecção dos padrões de metilação dos potenciais biomarcadores descritos no método de reivindicação 9.

12. Painel genético caracterizado pela detecção dos padrões de variantes polimórficos dos potenciais biomarcadores descritos no método de reivindicação 9.

13. Painel genético na predição de câncer ginecológico associado à síndrome metabólica caracterizada pela detecção de biomarcadores em variadas amostras biológicas, como soro, plasma, biópsia, tecido parafinizado, não excluindo outras, em associação com o diagnóstico clínico das pacientes a partir do método da reivindicação 9.

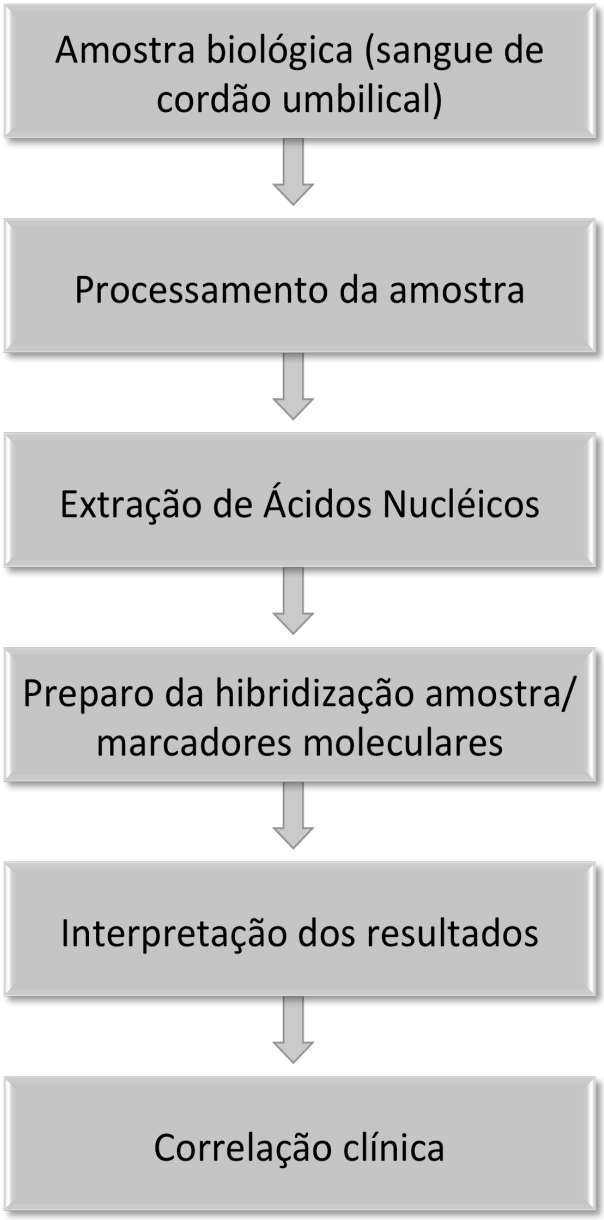
14. Painel genético na predição de câncer ginecológico associado à síndrome metabólica, principalmente em recém-nascidas com alteração do crescimento fetal, caracterizada pela detecção de potenciais biomarcadores conforme reivindicação 9.

PAINEL GENÉTICO NA PREDIÇÃO DE CÂNCER GINECOLÓGICO ASSOCIADO À SÍNDROME METABÓLICA

RESUMO

A invenção refere-se a um painel de marcadores genéticos que possuem potencial de diagnóstico e predição ao desenvolvimento de câncer ginecológico (mama, colo de útero, endométrio e ovário) em associação à síndrome metabólica, não excluindo outras doenças metabólicas, em recém-nascidas que apresentam alteração do crescimento fetal, para o diagnóstico precocemente do desenvolvimento destes fenótipos patológicos.

Figura 1


Amostra biológica (sangue de cordão umbilical)
Processamento da amostra
Extração de Ácidos Nucléicos
Preparo da hibridização amostra/ marcadores moleculares
Interpretação dos resultados
Correlação clínica

ANEXO B - COMITÊ DE ÉTICA

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: EXPRESSÃO IMUNOLÓGICA EM MULHERES COM HPV: COMPARAÇÃO ENTRE PORTADORAS DE LESÕES CERVICAIS E CÉRVICE NORMAL

Pesquisador: ana carla silva alexandre

Área Temática:

Versão: 1

CAAE: 24902313.4.0000.5208

Instituição Proponente: LABORATÓRIO DE IMUNOPATOLOGIA KEISO ASAMI

Patrocinador Principal: LABORATÓRIO DE IMUNOPATOLOGIA KEISO ASAMI

DADOS DO PARECER

Número do Parecer: 522.239

Data da Relatoria: 05/02/2014

Apresentação do Projeto:

O documento intitulado ¿EXPRESSÃO IMUNOLÓGICA EM MULHERES COM HPV: COMPARAÇÃO ENTRE PORTADORAS DE LESÕES CERVICAIS E CÉRVICE NORMAL¿ trata-se de projeto de dissertação de Mestrado em Ciências Biomédicas da aluna Ana Carla Silva Alexandre sob orientação do Prof. Dr. José Luiz de Lima Filho .

O projeto está adequadamente elaborado. Propõe-se a verificar a expressão imunológica de biomarcadores presentes no estroma cervical de mulheres portadoras de HPV de alto risco. A infecção provocada pelo papiloma vírus humano (HPV), é sexualmente transmissível, com efeitos que vão desde verrugas benignas para câncer invasivo. Alterações gênicas provocadas pelo vírus nas células cervicais, são mediadas por inúmeros biomarcadores que afetam diretamente o processo de replicação do HPV e inibição dos supressores tumorais.

Objetivo da Pesquisa:

Objetivo Primário:

Avaliar as diferentes modificações de sirtuinas 3, 4 e 5; citocinas IL 2, IL -06, IL-10, IL-18, interferons gama e beta nos variados graus de infecção por HPV numa instituição pública de referência para câncer cérvico-uterino de Pernambuco- Brasil .

Objetivos Secundários

Descrever a performance dessas citocinas através dos diversos graus de lesões;
Determinar a expressão das citocinas em amostras HPV positivo, sem lesão intraepitelial e as HPV negativa sem lesão intraepitelial;
Comparar a expressão das citocinas nas amostras com diagnóstico de NIC de alto grau e seus controles ;
Realizar genotipagem viral dos componentes da amostra.

Avaliação dos Riscos e Benefícios:

De acordo com os autores será realizado a coleta de material durante exame de Papanicolau de execução rotineira pelas mulheres. Este exame traz como risco o constrangimento por ser uma técnica invasiva com posição desconfortável, no entanto será garantido a mulher total privacidade e sigilo a respeito do (s) seu (s) diagnóstico. Quanto aos benefícios a mulher terá a garantia do resultado rápido com diagnóstico preciso de Papanicolau e específico de HPV, além de orientação e encaminhamento caso ocorra alguma alteração.

Comentários e Considerações sobre a Pesquisa:

Trata se de um estudo de caso controle envolvendo mulheres assintomáticas atendidas no Centro de Saúde da Mulher do Município de Pesqueira ¿ PE, por demanda espontânea para realização da citologia oncótica de rotina. Serão analisados 3 grupos totalizando 250 amostras: uma amostra de mulheres HPV negativas com exame citopatológico normal;mulheres HPV positivas com exame citopatológico normal e por último, amostra de mulheres HPV positivas com lesão de alto grau. Os critérios de inclusão e exclusão estão definidos.

Considerações sobre os Termos de apresentação obrigatória:

Foi anexado na plataforma carta de anuência do da Secretária de Saúde de Pesqueira e do Laboratório de Imunopatologia Keizo Asami. O orçamento será por conta dos pesquisadores. O Cronograma é compatível com os objetivos da pesquisa. O TCLE está adequadamente redigido.

Recomendações:

Não há.

Conclusões ou Pendências e Lista de Inadequações:

O presente estudo é relevante e traz grandes benefícios aos participantes. Está adequadamente redigido e tem metodologia apropriada.

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

O Colegiado aprova o parecer do protocolo em questão e o pesquisador está autorizado para iniciar a coleta de dados.

Projeto foi avaliado e sua APROVAÇÃO definitiva será dada, após a entrega do relatório final, na PLATAFORMA BRASIL, através de ¿Notificação ¿ e, após apreciação, será emitido Parecer Consubstanciado .

RECIFE, 05 de Fevereiro de 2014

Assinador por:
GERALDO BOSCO LINDOSO COUTO
(Coordenador)

Comitê de Ética em Pesquisa Envolvendo Seres Humanos - CEP-CCS-UFPE

UNIVERSIDADE FEDERAL DE PERNAMBUCO CENTRO DE CIÊNCIAS DA SAÚDE / UFPE-

Plataforma Brasil

PARECER CONSUBSTANCIADO DO CEP

DADOS DO PROJETO DE PESQUISA

Título da Pesquisa: SAÚDE DA MULHER: MARCADORES MOLECULARES PARA CÂNCER DE MAMA E CERVICAL

Pesquisador: Danyelly Bruneska Gondim Martins

Área Temática:

Versão: 2

CAAE: 35626514.5.0000.5208

Instituição Proponente: LABORATÓRIO DE IMUNOPATOLOGIA KEISO ASAMI

Patrocinador Principal: FUNDACAO DE AMPARO A CIENCIA E TECNOLOGIA - FACEPE

DADOS DO PARECER

Número do Parecer: 852.334

Data da Relatoria: 30/10/2014

Apresentação do Projeto:

Indicado na relatoria inicial.

Objetivo da Pesquisa:

Indicado na relatoria inicial.

Avaliação dos Riscos e Benefícios:

Indicado na relatoria inicial.

Comentários e Considerações sobre a Pesquisa:

Indicado na relatoria inicial.

Considerações sobre os Termos de apresentação obrigatória:

Indicado na relatoria inicial.

Recomendações:

Recomendação:

No orçamento, o equipamento pertence ao LIKA/UFPE, portanto não seria necessário incluir no orçamento, mas informar que o Laboratório permitirá o uso do mesmo.

Conclusões ou Pendências e Lista de Inadequações:

aprovado com recomendação.

Endereço: Av. da Engenharia s/nº - 1º andar, sala 4, Prédio do CCS
Bairro: Cidade Universitária **CEP:** 50.740-600
UF: PE **Município:** RECIFE
Telefone: (81)2126-8588 **E-mail:** cepccs@ufpe.br

Situação do Parecer:

Aprovado

Necessita Apreciação da CONEP:

Não

Considerações Finais a critério do CEP:

O Colegiado aprova o parecer do protocolo em questão e o pesquisador está autorizado para iniciar a coleta de dados.

Projeto foi avaliado e sua APROVAÇÃO definitiva será dada, após a entrega do relatório final, na PLATAFORMA BRASIL, através de "Notificação " e, após apreciação, será emitido Parecer Consubstanciado .

RECIFE, 30 de Outubro de 2014

Assinado por:
GERALDO BOSCO LINDOSO COUTO
(Coordenador)