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DIFFERENTIAL GENE EXPRESSION AND PATHWAYS ASSOCIATED WITH THE ANGIOGENESIS IN ESOPHAGEAL NASOPHARYNGEAL CANCER[1]

DIFERENCIAÇÃO DA EXPRESSÃO E VIAS ASSOCIADAS COM A ANGIOGÊNESE EM CÂNCER DE ESÔFAGO E NASOFARINGE

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ABSTRACT

Cancer is a disease caused by molecular disorders associated to the mechanisms by which cell division is controlled. In this sense, diseases were analyzed with the purpose of clarify the molecular pathways behavior and mechanisms associated with genome stability in cancer. The goal this study was to better elucidate the role of angiogenesis pathways in esophageal and nasopharyngeal cancer using quantitative methods to determine gene expression, activity and relative diversity. The results obtained suggested that the enzymes of the matrix metalloproteinases family are associated with the development and progression of cancer in relation to esophageal and Nasopharynx. These findings may contribute to improve the elucidation of the mechanisms associated with the angiogenesis in the evolution and differentiation in cancer cases considered by us.

Keywords: MMPs, vascularization, activity.

RESUMO

O câncer é uma doença causada por distúrbios moleculares associados aos mecanismos pelos quais a divisão celular é controlada. Nesse sentido, foram propostos modelos com o objetivo de esclarecer o comportamento das vias moleculares e os mecanismos associados à estabilidade do genoma no câncer. O objetivo deste estudo foi observar a expressão das vias de angiogênese no câncer esofágico e nasofaríngeo usando métodos quantitativos para determinar a expressão gênica, atividade e diversidade relativa. Os resultados obtidos sugeriram que as enzimas da família das metaloproteinases da matriz estão associadas ao desenvolvimento e progressão do câncer em relação ao esofágico e à Nasofaringe. Esses resultados podem contribuir para melhorar a elucidação dos mecanismos associados à angiogênese na evolução e diferenciação em casos de câncer.

Palavras-chave: *MMPs, vascularização, atividade.*

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INTRODUCTION

Tumor angiogenesis is associated with growth of blood vessels and capillaries which increase during the development of cancer. This mechanism is essential for tumor nutrition and migration of cancer cells to healthy tissues (WANG et al., 2015). Angiogenesis can be understood through three distinct processes (CARMELIET et al., 2017, GOODWIN, 2007). The first step is associated with degradation of basement membrane (vascular matrix) and formation of a vascular bud, which is the key process of metastasis and tumor invasion (NISHIDA et al., 2006). This degradation occurs through activation of metalloproteinases enzymes that are responsible for disorganizing the matrix. In the second process occurs the migration of endothelial cells, which will compose the vessel wall. The latter process is the maturation, formation and inhibition of growth, which will result in complete formation of vessel and in formation of angiogenesis (NISHIDA et al., 2006; CARMELIET et al., 2017).

Several proteins have been identified as activators and inhibitors of angiogenesis. Vascular endothelial growth factor (*VEGF*) is a protein that promotes the growth of new vessels (NISHIDA et al., 2006). In continuous tumors, this protein stimulates growth and maturation of vessels. For this reason, mutated cells may develop and spread to other tissues in cancers that express this protein (CROSS; WELSH, 2001; NISHIDA et al., 2006). The basic fibroblast growth factor (*bFGF*) is an angiogenic factor that provides resistance to extracellular matrix (FOLKMAN, 1995). Tumor necrosis factor (*TNF*-α) is one of several proteins expressed in formation of angiogenesis and is involved in inflammatory processes (CROSS; WELSH, 2001).

Cancer-related angiogenesis is an important subject of study because it is an essential mechanism for tumor development (ZIYAD; IRUELA-ARISPE, 2011). Angiogenesis contributes nutritionally to tumor growth and is a channel for proliferation of neoplastic cells, necessary for formation of metastases (NISHIDA et al., 2006, CARMELIET et al., 2017). Senger and collaborators related proteins with activation or inhibition of angiogenesis pathway and highlight the importance of develop studies about this pathway (NISHIDA et al., 2006; SENGER et al., 1983). Therefore, the aim of this study is to investigate the activation of angiogenesis pathway in esophageal and nasopharyngeal cancer using quantitative methods to determine gene expression, activity and diversity relative.

MATERIAL AND METHODS

MICROARRAY DATA SELECTION

Transcriptome data of esophageal and nasopharyngeal cancer was selected using the Gene Expression Omnibus data base (GEO). Gene expression data were obtained by the Affymetrix Gene Chip HU133 microarray technique, containing 26,000 genes approximately:

1. GSE17351: control and cancer esophageal cell samples from 5 male patients of Japanese origin with ages ranging from 51 to 76 years (GSE17351, 2009).

2. GSE12452: 10 normal nasopharyngeal cell samples and 31 nasopharyngeal carcinoma samples, produced by cell culture and differentiated according to the protocol published in the GSM312896 in 2008 (GSE12452, 2008).

To establish a biological analysis pattern among the data series the samples used were normalized using the robust medium for multiple chips (RMA) (BAÚ, 2016).

ANGIOGENESIS PATHWAYS

Angiogenesis pathways were extracted from the Reactome database, an open-source repository which stores pathways of various biological processes and tools for visualization, interpretation and analysis of pathways (CROFT et al., 2013; FABREGAT et al., 2015). The data were selected according the functionality and development of angiogenesis which occurs during the three processes established above. Angiogenesis pathways used in this study are shown in the results section.

STATISTICAL ANALYSIS

The arithmetic mean was used to group the samples and two groups were used to perform the analysis: esophageal (experimental versus control) and nasopharyngeal (experimental versus control). Considering the average of the samples, the log fold change (LogFC) was used to calculate each gene differentially expressed by the log of the ratio between the experimental and control tissue. The software R uses statistically significant data involving Baysian and Tstudent methods (BAÚ, 2016). Differences between experimental and control data were considered statistically significant for values <5% and LogFC > 2 (considering base 2). To execute the analysis of Reactome pathways the Via Complex software was used to calculate the relative activity (ATR) and the relative diversity (DIV). The methodology used to calculate ATR and DIV (p-value < 5%) is described in more detail by Simão and co-authors (SIMÃO et al., 2012).

RESULTS AND DISCUSSION

FOLD CHANGE

According to the method used to calculate gene expression using fold change, 43 genes overexpressed in cases of esophageal cancer and 50 genes overexpressed in cases of nasopharyngeal cancer were observed (Table 1). The significant test was calculated by T-student test with significant of the 5%. Among these, 6 genes were mutually expressed in two diseases: *COL1A1*, COL1A2**, MMP1***, MMP12***** and *SPP1******, in addition to the intersection of *ADAM* and *MMP* families.

Table 1 - Differential expression genes for esophageal and nasopharyngeal cancer using fold change calculation (p-value < 5%, T-student test).

Esophageal			Nasopharyngeal		
Gene Symbol	**LogFC**	**P-value**	**Gene Symbol**	**LogFC**	**P-value**
*MMP1 ****	5.55	0.0349	*CXCL11*	3.84	0.0000
*MMP12*****	4.04	0.0455	*FN1*	3.20	0.0000
CST1	3.82	0.0401	*PTGS2*	3.12	0.0000
*COL1A1**	3.75	0.0238	*MMP1****	3.03	0.0009
CTHRC1	3.70	0.0076	*CXCL10*	2.92	0.0000
MMP13	3.42	0.0038	*POSTN*	2.88	0.0008
NELL2	3.29	0.0094	*LAMB1*	2.71	0.0000
*SPP1******	3.28	0.0302	*VASH2*	2.66	0.0002
WDR66	3.13	0.0139	*ADAM23*	2.63	0.0006
INHBA	3.11	0.0401	*LHX2*	2.63	0.0000
MFAP2	2.75	0.0113	*COL1A1**	2.62	0.0000
LAMC2	2.68	0.0076	*NREP*	2.62	0.0001
KIF14	2.59	0.0076	*COL1A2***	2.57	0.0000
PLAU	2.58	0.0031	*CHI3L1*	2.47	0.0000
FABP6	2.57	0.0323	*CXCL9*	2.46	0.0000
MMP11	2.56	0.0105	*DTL*	2.42	0.0000
DNMT3B	2.53	0.0084	*COL4A1*	2.39	0.0000
HOXB7	2.45	0.0316	*SPP1******	2.37	0.0099
CDH3	2.34	0.0232	*C6orf141*	2.34	0.0000
ADAMTS2	2.34	0.0407	*HOXC6*	2.33	0.0000
SERPINH1	2.30	0.0078	*CACNA2D1*	2.33	0.0000
NETO2	2.26	0.0188	*TNFAIP6*	2.29	0.0000
GALNT6	2.25	0.0308	*COL3A1*	2.27	0.0001
JPH1	2.19	0.0186	*GAD1*	2.26	0.0000
BUB1	2.18	0.0188	*SULF1*	2.24	0.0001
APOC1	2.16	0.0345	*RAD51AP1*	2.23	0.0000
*COL1A2***	2.16	0.0447	*PBK*	2.22	0.0000
TPX2	2.10	0.0068	*GREM1*	2.22	0.0002
AURKA	2.08	0.0118	*TYMS*	2.22	0.0000
CDC25B	2.03	0.0094	*MMP12*****	2.20	0.0003
UBE2C	2.03	0.0057	*COL5A2*	2.20	0.0000
MFHAS1	2.03	0.0238	*COL4A2*	2.19	0.0000
ITGA6	2.03	0.0228	*GZMB*	2.18	0.0000
CDKN3	2.02	0.0057	*CCNE2*	2.18	0.0000
PGBD5	2.01	0.0235	*PRRX1*	2.17	0.0002
PITX2	2.01	0.0267	*SYNPO2*	2.15	0.0005
			CCL2	2.14	0.0004
			CCNB1	2.12	0.0000
			CCL8	2.12	0.0002
			ATAD2	2.08	0.0000
			KIF23	2.07	0.0000
			NUF2	2.06	0.0000
			MAD2L1	2.02	0.0000
			HELLS	2.00	0.0000

*genes of consensus between the two diseases.

The *COL1A1* and *A2*, collagen type 1 alpha 1 and 2 chain genes act in formation of directed extracellular matrix and stimulate angiogenesis. The genes characterized by *MMP1* and *MMP12*, matrix metallopeptidase 1 and 12, are part of the family of metalloproteinase enzymes, which has as main characteristic the regulation of extracellular matrix. As a consequence of this extracellular matrix degradation the increased expression of these proteins is associated with the development of angiogenesis (JUCÁ et al., 2008). The combined action of this enzymes could degrade the entire matrix. According to the function of this family of enzymes it is possible to subdivided them in groups as, for example, collagenases, gelatinases, stromelysins and membrane metalloproteinases (SWEENEY et al., 2008). The inhibition of proteins from *MMPs* family was created using proteins that stimulate the equilibrium between destruction and formation of the new matrix. These proteins are entitled tissue inhibitors of metalloproteinases (TIMPs) and have a important function against formation of vessels and capillaries in various types of tumor (WOJTOWICZ-PRAGA; DICKSON; HAWKINS, 1997). In addition, *SPP1* (Secreted Phosphoprotein 1) protein is involved in the binding of osteoclasts to the bone matrix located in the cell membrane; therefore, this protein may be related to the development of angiogenesis (ROWE et al., 2014).

RELATIVE ACTIVITY AND DIVERSITY

According to the methodology used in this study, 9 pathways available in the Reactome database were investigated (Table 2). Functionality and development of these pathways are associated with angiogenesis in cancer formation. In table 2 is shown the activity and relative diversity calculation and the comparison of pathways expression in esophageal and nasopharyngeal cancer with their respective controls.

Four pathways with altered activity ($p<0.05$) were observed for the consensus of cancers analyzed: collagen degradation, basigin interactions, activation of matrix metalloproteinases and degradation of the extracellular matrix. The large activity of these pathways contributes to development and progression of cancer. Although, the contribution is different for each pathway there is a relation between them. Collagen degradation pathway requires enzymes and activation of matrix metalloproteinases to occur the collagen breakage. Basigin interactions pathway stimulates the synthesis of *MMPs*, which is present in the unregulated form in many diseases. In addition, this pathway induces angiogenesis through vascular endothelial growth factor (*VEGF*).

Degradation of extracellular matrix pathway is associated with cancer induction. Extracellular matrix is a dynamic structure in constant process of remodeling its components, which admit cellular differentiation, branched morphogenesis, angiogenesis, bone remodeling and wound repair. Occurrence of anomalies in the extracellular matrix could cause proliferation and invasion of defective cells and failure of apoptosis, which could lead to formation of cancer (LU et al., 2011).

Tabela 2 - Results of relative activity (ATR) and relative diversity (DIV) for both cancers of the esophagus and nasopharynx.

		Esophageal				Nasopharyngeal			
Name Pathway	**Genes**	**ATR**	**P-value**	**DIV**	**P-value**	**ATR**	**P-value**	**DIV**	**P-value**
Collagen degradation	37	0.528	*	0.500	NS	0.510	*	0.500	NS
Activation of Matrix Metalloproteinases	25	0.521	*	0.500	NS	0.505	*	0.500	NS
Basigin interactions	21	0.519	*	0.500	NS	0.508	*	0.500	NS
Regulation of Insulin	32	0.501	NS	0.500	NS	0.506	*	0.500	NS
Interleukin 4 and 13 signaling	109	0.502	NS	0.500	*	0.502	*	0.500	*
Degradation of the extracellular matrix	104	0.507	*	0.500	NS	0.503	*	0.500	NS
Metabolism of Angiotensinogen to Angiotensins	16	0.503	NS	0.501	NS	0.500	NS	0.500	NS
Signaling by VEGF	326	0.500	NS	0.500	NS	0.501	NS	0.500	NS
VEGFR2 mediated vascular permeability	27	0.500		0.500		0.499		0.500	

* P-value <5%, NS: no significant.

Activation of matrix metalloproteinases pathway is related to the process described above. These process could be understand as an activation pathway associated to *MMPs* in which these enzymes normally participate in regulation and remodeling of extracellular matrix (MEC), which are important in many normal processes of the organism. However, a large expression of these proteins involves the participation of this pathway in many diseases. In cancer it contributes to invasion of cancer cells and metastases. Because of MEC regulation, this pathway directly contributes to degradation of extracellular matrix pathway. In cases in which occur a large expression of *MMPs* and these enzymes start to degrade the MEC in a deregulated way, degradation of extracellular matrix pathway could cause cell proliferation as a consequence of failure of apoptosis. Degradation of extracellular matrix pathway could cause cell proliferation as a consequence of failure of apoptosis in cases in which occur a large expression of *MMPs* and these enzymes start to degrade the MEC in a non-controlled way.

Collagen degradation and activation of matrix metalloproteinases pathway have as main characteristic the possibility of degrade collagen through enzymes of metalloproteinases family. MMPs has an important part in embryonic development, such as growth and remodeling. However, excessive and uncontrolled expression of *MMPs* is associated with many diseases, such as arthritis, multiple sclerosis, tumor progression and cancer (HADLER-OLSEN et al., 2010).

Basigin interactions pathway is related to basigin glycoprotein and correlated to several phenomena, such as differentiation and development. However, its main characteristics is to stimulate the synthesis of metalloproteinases and induce the process of angiogenesis through stimulation of VEGF production (LI; JUAN, 2007).

Insulin regulation pathway control the levels of insulin release in the body through blood glucose levels. Insulin release occurs through pancreatic beta cells and glucose catabolism (WIEDERKEHR; WOLLHEIM, 2006). According to Escudero et al. (2017) this pathway operates in the pro-angiogenic state and is potentiated by the generation of vascular growth factors.

Interleukin 4 and 13 signaling pathway has the function of signalize interleukin-4 (*IL-4*) and regulate the immune response. One of its functions is to mobilize mediators of cell growth, resistance to apoptosis and, activation and differentiation of genes (NELMS et al., 1999). Interleukin-13 is an immunoregulatory cytokine with functional properties similar to *IL-4* (HERSHEY, 2003).

Metabolism of angiotensinogen for angiotensins pathway is active synthesized and secreted mainly by the liver. Renin protein, is secreted in bloodstream by juxtaglomerular cells of the kidney, in response to a decrease of blood pressure. Also, it can bind membrane-localized (pro) renin receptor which increases its catalytic activity. The angiogenesis is activated after cleavage and development of active angiotensinogen (FYHRQUIST; SAIJONMAA, 2008).

Signaling by *VEGF* and *VEGFR2* mediated vascular permeability are essential pathways for vascular regulation and development. These pathways act during activation of collagen degradation and activation of matrix metalloproteinases pathways. Also, abnormal VEGF function is associated with inflammatory diseases including atherosclerosis, and hyperthyroidism (RODRÍGUEZ; MORRISON; OVERALL, 2010).

CONCLUSION

Considering the results obtained in the analysis of change in gene expression and activity and relative diversity of pathways is concluding that enzymes of matrix metalloproteinases family are associated with development and progression of cancer in relation to esophageal and nasopharynx. *MMP1/12* genes show a large change of expression in cancer samples. Also, it was observed that the pathways containing these genes appear with large activity in cancer. Thus, there is a possibility use it as a therapeutic target, as well as their respective pathways. To induce inhibition of *MMP*s it is suggested to use nanocapsules, such as polymer nanocapsules which can be used to encapsulate proteins in order to increase efficiency of cancer treatment.

In some studies authors used a “nano-quercetin to arrest mitochondrial damage and *MMP-9* upregulation during prevention of gastric inflammation induced by ethanol in rat”, which suggests the use of nanocapsules for treatment of diseases. The use of these nanocapsules associated with the specific proteins could contribute to the effectiveness of treatments in combating development of angiogenesis and, consequently, fight against cancer.

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