A protein interaction model, based on angiogenic gene expression analysis, in a larynx carcinoma case

MARIAN CONSTANTIN (1)(2)*, BOGDAN IANCU (3), DANIELA IANCU (3)(4), DOREL AUGUSTIN MANU (5) and LIGIA GABRIELA GHETEA (2)

1 Romanian Academy, Institute of Biology, 296 Splaiul Independenței, Bucharest, Romania
2 University of Bucharest, Faculty of Biology, Department of Genetics, Bucharest, Romania
3 National Institute of Legal Medicine ‘Mina Minovici’, Laboratory of Genetics, Bucharest, Romania
4 University College London, Centre for Nephrology, Royal Free Hospital, London, United Kingdom
5 Ilfov County Emergency Hospital, Otorhino-Laryngology Clinic, Bucharest, Romania

*Corresponding author
Marian Constantin
marian.constantin@ibiol.ro, cvgmarian@gmail.com

Keywords. VEGF, IL8, PDGF, MMP2, TGB1, ANGPT2, FGF2, TIE2, CXCL12, IL12A, BAI1, CXCL10, IFNG, TNFSF15, String 9.1.

Summary

The aim of this study was to propose an original approach for the analysis of tumorigenic factors expressed in a specific tumor: a theoretical framework of angiogenic and angiostatic protein interactions, in order to find the way in which the type and amount of some proteins expression affect the expression and activity of other proteins involved in a tumoral process. This study was conducted using biological material (tumoral and peritumoral tissue fragments) from a 71 years old male patient diagnosed with laryngeal carcinoma. The expression levels of several angiogenic genes have been analyzed by qPCR (relative quantification, RQ). Based on these results, we built a protein functional interaction network model, using String® protein networking software. Such a model could offer a more integrative image on the particularities and intensity of a tumoral process in progress. Also, it could indicate potential therapeutic targets (key proteins), for a more efficient, personalized therapy.

Introduction

Angiogenesis is the biological process by which new blood vessels are formed from the pre-existing ones, by ‘budding’. This process is very active in embryos, during organ morphogenesis; in an normal adult organism its intensity is significantly reduced, being limited to wound healing and to proliferative phase of the uterine cycle (Billioux, 2005, Ruddon, 2007). Angiogenesis is encountered also in pathological conditions as rheumatoid arthritis, atherosclerosis, endometriosis, diabetic retinopathy, psoriasis, tumorigenesis (Billioux, 2005).

In the initial tumoral stages, when the tumors are smaller than 1-2 mm (Wicki, 2007, Ruddon, 2007), they do not need vascularization, receiving nutrients and oxygen by diffusion. In these avascular or prevascular stages (Billioux, 2005), in tumors there is an equilibrium between proliferation and apoptosis of tumoral cells, the tumor volume, the required amount of nutrients and oxygen remaining quasi constant.

When the cell proliferative rate increases, the cellular mass needs significantly increased amounts of nutrients and oxygen. If these requirements are not met by diffusion, the growing tumor enters in a hypoxic stage, and begins to secrete the hypoxia inducible factor (HIF); this factor is an efficient chemoattractor for M2 macrophages which produce and release inflammatory and angiogenic- (FGF, VEGF, TGF, MMP) and angiostatic factors (TSP) (Balkwill et al., 2005). Besides HIF, the tumor secretes
numerous other proteins (CSF, TGFB, CCL, MIF, and others) that lead to the onset of the angiogenic switch (Wicki, 2007).

Formation of new blood vessels (angiogenesis) is a key-process for tumor progression and metastasis. In the absence of angiogenesis, the tumor cannot continue its growth over 1-2 mm (because of the absence of nutrients and oxygen), it suffers apoptosis or necrosis. In growing tumors, endothelial cells that will form rudiments of new blood vessels, may proliferate 20 to 2000 times faster than normal tissue endothelium of an adult organism. Tumor vascular system is structurally and functionally abnormal, being disorganized, tortuous and dilated, leading to chaotic blood flow and variable regions of hypoxia. Although full vascularization of the tumor does not occur, it does provide nutrients for its growth. Thus, angiogenesis is an important process involved in converting in situ carcinomas to aggressive malignant tumors. Blocking the process could inhibit or significantly slow this conversion.

Materials and methods

The tumoral- and peritumoral tissue fragments have been obtained from a primary tumor of a 71 years old male patient diagnosed with laryngeal neoplasm (T3N2bM0); the patient has not received radio-/chemotherapy before surgical remove. He was operated on April 2011, at Oto-Rhyno-Laryngology Clinic of the Ilfov County Emergency Hospital, in Bucharest. The histopathological analysis of the removed tumoral tissue showed a keratinized squamous cell carcinoma with metastasis in one latero-cervical lymph node.

The patient has given an informed written consent for his biological samples (blood and tissue fragments surgically removed) to be used for cytological and molecular investigations, and to report and publish the obtained data, considering the requested confidentiality on his identity. The research protocol was approved by the Ethics Committees of the participant institutions. The study was performed in accordance with the Declaration of Helsinki.

Total RNA was isolated from 100mg of tumoral- and 100mg of peritumoral (used as control) tissue, using the NucleoSpin RNA II-Total RNA Isolation Kit (ref.740955.50, MACHEREY-NAGEL GmbH & Co. KG, 52355 Düren, Germany). The concentration and purity of the total RNA obtained in the two samples was analyzed by spectrophotometry (at 260/280nm, and 260/230nm).

The reverse transcription of total RNA was performed with the High-Capacity cDNA Reverse Transcription Kit (cat.no.4368814, Applied Biosystems, Foster City, CA 94404, USA); 900 ng of total RNA were reverse transcribed, and the reaction efficiency was considered to be 100%, as stated by the manufacturer.

For the quantitative PCR (qPCR) reaction, TaqMan® Array 96-Well Plates Gene Signature for Human Angiogenesis (cat.no.4414071, Applied Biosystems, Foster City, CA 94404, USA) were used; these plates contain 92 genes involved in angiogenesis, and 4 housekeeping genes: 18S (for RNA 18S), GAPDH (for glyceraldehyde-3-phosphate dehydrogenase), HPRT1 (for hypoxanthine phosphoribosyltransferase 1) and GUSB (for β-glucuronidase). The TaqMan® Gene Expression Master Mix (2x) (cat.no.4369016, Applied Biosystems, Foster City, CA 94404, USA) was used; the final reaction volume was of 20 µl, from which 10 µl of master mix, and 10 µl of sample containing 25 ng of cDNA template. The amplification reaction was performed on a Real-Time PCR 7500 (Applied Biosystems) device; the reaction conditions were: 95°C-10 min; 40 cycles of 95°C-15 sec, 60°C-1 min, run in standard mode.

The data were analyzed using the DataAssistTM Software designed for sample comparison based on the comparative Ct (ΔΔCt) method (Schmittgen & Livak 2008) for calculating the relative quantification (RQ) parameter, regarding gene expression levels in two different samples. The housekeeping genes considered as endogenous control were 18S and GAPDH. Based on the gene expression results, 16 genes were chosen for further analyses. The functional relationships between the products
of these genes were identified, and the corresponding interaction networks were built using String® 9.1 program, available on www.string-db.org (Jensen et al., 2009). This program allows the searching of a specific protein or a protein list from one or of from multiple species, using their names or the amino acid sequences. When specified, the program builds the orthology tree of a gene or the interaction network for a specific protein or for a protein list. The interaction networks can be built by choosing among several interactions types, by required confidence levels, based on literature and experimental data. When needed, the program can seek and add the more appropriate interactors (i.e. protein partners) to the main network, their number being customizable.

In addition, String® can group the interactors upon their interaction affinities to several groups, using the MCL (Markov CLustering) algorithm, which detects clusters based on stochastic flow simulation (Dongen, 2000), i.e. groups proteins upon their functional affinities. The MCL has been proven to be effective at clustering biological networks and it accepts an inflation parameter, which can take values among 1 and 5: 1 for less functional affinities and 5 for more functional affinities. String® also highlights the nodes (proteins) that interacts for specified biological processes, or the proteins which are located in the same cellular compartment or in the extracellular space and can give some information about each node, including their conformational structure. It allows for the networks to be saved in several formats, including vector scalable graphics (svg), which is a vector file type format, or portable network graphics (png), which is a raster file type format.

When the networks in Figures 2 and 3 were built, we used the maximum required confidence (0.9), 10 interactors added (KDR, FLT1, TIMP2, FGFR1, TEK, TGFBR1, FLT4, TGFBR2, PDGFRB şi NRP1) and all the predictive methods used by the program: Neighborhood, Gene Fusion, Co-occurrence, Co-expression, Experiments, Databases, Textmining).

Two networks were built, one of them displaying the interactions among the 16 analyzed proteins (Figure 2 and Table II) and the second displaying the nodes interactions after introducing the MCL algorithm (Figure 3 and Table II). For creating the network in Figure 3, we used the maximum inflation value, 5. Any of the networks contains 26 proteins and the interactions among them.

Table 1. The gene expression (mRNA) levels, quantified by qPCR, expressed as CT (cycle threshold) values, in tumoral (T) tissue versus peritumoral (Pt) tissue and the gene expression (mRNA) levels in tumoral tissue (T) versus peritumoral tissue (Pt): relative quantification (RQ) values. Red boxes – overexpression in tumoral tissue; green boxes – underexpression in tumor; black boxes – similar expression in both tissues.

<table>
<thead>
<tr>
<th>No.</th>
<th>Genes</th>
<th>Angiogenic (+) / angiostatic (–) effect of the gene product</th>
<th>CT values</th>
<th>Pt</th>
<th>T</th>
<th>RQ</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>VEGF4</td>
<td>+</td>
<td>27.003</td>
<td>27.03</td>
<td>0.9743</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>VEGFB</td>
<td>+</td>
<td>24.729</td>
<td>26.08</td>
<td>0.4107</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>VEGFC</td>
<td>+</td>
<td>32.115</td>
<td>29.564</td>
<td>4.6557</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>IL8</td>
<td>+</td>
<td>32.335</td>
<td>26.171</td>
<td>56.9648</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>PDGFβ</td>
<td>+</td>
<td>28.538</td>
<td>26.603</td>
<td>3.0377</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>MMP2</td>
<td>+</td>
<td>26.654</td>
<td>23.613</td>
<td>6.5587</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>TGFβ1</td>
<td>+</td>
<td>28.717</td>
<td>23.778</td>
<td>24.3694</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>ANGPT2</td>
<td>+</td>
<td>33.02</td>
<td>30.724</td>
<td>3.9014</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>FGFR2</td>
<td>+</td>
<td>31.456</td>
<td>28.319</td>
<td>6.9888</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>TIE1</td>
<td>+</td>
<td>28.95</td>
<td>28.063</td>
<td>1.4692</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>CCXL2</td>
<td>+</td>
<td>25.959</td>
<td>23.623</td>
<td>4.0111</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>IL12A</td>
<td>–</td>
<td>36.424</td>
<td>31.411</td>
<td>25.652</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>BAII1</td>
<td>–</td>
<td>37.118</td>
<td>32.276</td>
<td>22.7848</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>CCX110</td>
<td>–</td>
<td>32.622</td>
<td>22.142</td>
<td>113.6251</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>FNG</td>
<td>–</td>
<td>39.089</td>
<td>27.716</td>
<td>2107.0371</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>TNNFSF15</td>
<td>–</td>
<td>36.039</td>
<td>28.015</td>
<td>206.7868</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1. Gene expression levels in tumoral- versus peritumoral tissue: the relative quantification (RQ) values (presented as log2 values) for the analyzed genes. Black – angiogenic genes; light gray – angiostatic genes.
Results and discussions

Results

We analyzed the expression levels for 16 genes whose products are involved in angiogenic processes, among which 11 have angiogenic effects and 5 have angiostatic effects. Table I displays the gene expression as CT values and RQ values. The red boxes mark the overexpressed genes (those having RQ values over 2) in the tumor, in comparison to the non-tumoral (control) tissue; the black box denotes the comparable expression in tumoral and in control tissues (that having RQ values among 0.500 and 1.999), and the green boxes point the underexpressed genes in tumor, in comparison with the control tissue (those having RQ values under 0.499).

VEGFA sustains vascular permeability, allowing tumoral and non-tumoral cells to extravasate (Papetti & Herman, 2002); it has chemoattraction activity over monocytes, protects neurons (Tammela et al., 2005), suppress endothelial cells apoptosis and is the most potent indutor for angiogenic processes (Balkwill et al., 2012, Ferrara, 2004). The expression of VEGFA mRNA is slightly decreased in the tumoral tissue.

VEGFB is stimulating the soreness-associated angiogenesis (Tammela et al., 2005). The VEGFB gene is underexpressed in tumor.

VEGFC is secreted as a response reaction to the inflammatory processes (Jussila & Alitalo, 2002, Tammela et al., 2005) and is an inductor for vascular endothelial cells mitosis and migration (Tammela et al., 2005). It sustains angiogenesis (Jussila & Alitalo, 2002), lymphangiogenesis (Balkwill et al., 2012, DeMei et al., 2013, Jussila & Alitalo, 2002, Tammela et al., 2005) and also lymph nodes invasion and metastasis (Tammela et al., 2005). The VEGFC gene expression in tumor is higher than in the peritumoral tissue.

IL8 has chemoattraction function over neutrophils and lymphocytes, stimulating tumor cell proliferation, migration, invasion and angiogenic processes (Jo et al., 2013, Savage et al., 2004, Yu et al., 2013); it is also a marker for inflammatory processes (Qin et al., 2012). Its corresponding gene is overexpressed in tumoral tissue.

PDGFB is expressed by endothelial cells, by megakaryocytes and by neurons. When it is expressed by tumoral cells, it induces new blood cells stabilization (Andrae et al., 2008). The PDGF gene is overexpressed in tumoral tissue.

MMP2 is a matrix metalloproteinase and it is involved in extracellular matrix degradation, sustaining tumor cells and endothelial cells invasivity, and, by these actions, being positively involved in angiogenesis (van Hinsbergh & Koolwijk 2008) and in metastases formation (Wang et al., 2008). The MMP2 gene is overexpressed in the tumoral tissue.

TGFB1 sustains cell proliferation and differentiation. It also functions as a regulator of the immune system; it is positively involved in fibronectin and proteoglycans synthesis into the extracellular matrix (Horiguchi et al., 2012, Jiang et al., 2012). In the early stages of tumor development, TGFB1 inhibits tumor growth, but in the late stages, it sustains tumor development (Witsch, 2010). The TGFB1 gene is overexpressed in the tumoral tissue.

ANGPT2 is overexpressed in inflammation affected locations and in several neoplasias. It has vascular reshape activity (Thurston & Daly, 2012). The level of ANGPT2 mRNA in tumor is comparable to that from the control tissue.

FGF2 has stimulatory activity on the tumoral cells. It also diminishes proteolysis and sustains endothelial cells differentiation and migration (Husseman et al., 2012, Mori et al., 2013), promoting their organization into vascular structures (Sahni et al., 2008). The FGF2 gene is overexpressed in tumor.

In hypoxia, TIE2 sustains angiogenic processes, following its interaction with protein tyrosine phosphatase b (Sato et al., 1998, Yacyshyn et al., 2009). The mRNA of TIE2 gene is expressed at comparable levels in tumoral and control tissue.

By interaction with several cell types, CXCL12 has a stimulatory activity for tumor cell proliferation, migration (Rainczuk et al., 2012), metastasis (Balestrieri et al., 2008) and invasivity (Vindrieux et al., 2009). CXCL12 gene appears overexpressed in tumoral tissue.
IL12A has an angiostatic and antitumoral activity by cytotoxic T-cell activation. It also stimulates the expression of CXCL9, CXCL10 and IFNG genes (Airoldi & Ribatti, 2011, (Chen et al., 2009). IL12A gene is overexpressed in the tumoral tissue.

BAI1 protein is usually expressed in platelets, in liver and brain cells, inhibiting angiogenic processes (Duda et al., 2002, Dupuy & Caron, 2008, Kang et al., 2006, Koh et al., 2004, Nishimori et al., 1997). Its expression is sustained by TP53, so, when the latter is silenced, BAI1 protein is underexpressed (Duda et al., 2002). The BAI1 gene is overexpressed in tumor.

CXCL10 has angiostatic (Balestrieri et al., 2008), (Sato et al., 2007) and anti-tumor activity and it is chemoattractant for regulatory T-cells (Rainczuk et al., 2012). The CXCL10 gene is significantly overexpressed in the tumoral tissue.

IFNG has antitumor, apoptotic and angiostatic activity, also stimulating antiviral cell immune response (Papetti & Herman, 2002). Its corresponding gene is significantly overexpressed in tumoral tissue.

TNFSF15 has a mediatory function for inflammatory processes and for the cell growth. Also, it sustains NF κB transcription factor and c jun kinase (Haridas et al., 1999), it inhibits angiogenic processes (Deng et al., 2012, Haridas et al., 1999, Hou et al., 2005) and has a suppressive action on colon cancer cells (Haridas et al., 1999). Its overexpression in normal ovary vascular epithelium leads to angiogeneis inhibition. When VEGF starts to be overexpressed, TNFSF15 expression tends to be suppressed and angiogenic processes are stimulated (Deng et al., 2012). TNFSF15 gene is significantly overexpressed in the tumoral tissue.

Discussion
The analyzed case is a laryngeal neoplasm (T3N2bM0), in a 71 years old male patient; a primary tumor, non radio-/chemotherapy subjected before surgical remove. The hystopathological analysis showed a keratinized squamous cell carcinoma with metastasis in one latero-cervical lymph node. The associated diagnoses of this patient were chronic tonsillitis (so, chronic inflammation!) and weight loss. Currently, this patient is alive, and with no relapse.

By building and analyzing the protein functional relationship networks, we look for clues to explain this very positive situation, regarding the expression of some angiogenic and angiostatic genes and of their products.

VEGFs are potent angiogenic factors; in this case, VEGFA and VEGFB genes appear underexpressed, and VEGFC gene is overexpressed. VEGFA has the most protein partners (16 protein partners, among which, 11 are included in its affinity group, as shown in Figure 3) in the functional interaction network presented in Figure 2. Among the 16 interactors of VEGFA, 9 were included for mRNA assay in our study and 7 were added by String®.

VEGFC, PDGFB, MMP2, ANGPT2, FGF2 and CXCL12 proteins are overexpressed; TIE2 is expressed at comparable levels as in the peritumoral tissue; all these proteins have cumulative effects in the angiogenic switch, in the assessed tumor.

IL8 is overexpressed, indicating the presence of inflammatory processes inside the tumor. According to the networks shown in Figures 2 and 3, IL8 activity is sustained by VEGFA, which is underexpressed, and CXCL12, which is overexpressed. CXCL12, VEGFA and IL8 have a stimulating activity on MMP2 expression. Since VEGFA is underexpressed, it has little or no influence on MMP2 expression. The only proteins which can positively affect MMP2 expression level are CXCL12 and IL8, which seems to be enough for MMP2 to be 6.5-times overexpressed in the tumoral tissue, sustaining the release and migration of tumoral cells from the primary tumor (Wang et al., 2008) and to establish the metastase into the laterocervical lymph node, as occurs in the analyzed case.

TGFB1 has angiogenic activity and it is overexpressed in the tumoral tissue fragment, sustaining FGF2 expression. Even if FGF2 is specifically involved in growth and angiogenic processes, its 6.9-times overexpression in tumoral tissue fragment seems to have some influence over the CXCL12 expression. It seems to inhibit the expression of CXCL12 gene, which appears
to be 'only' 4-times overexpressed in the tumoral tissue, probably also due to FGF2 activity.

Interestingly, all the assessed proteins having angiostatic effects are overexpressed. IL12A is secreted by dendritic cells and by macrophages and forms IL12 heterodimers with IL12B, which is secreted by activated macrophages. IL12 has a stimulatory action over IFNG production and activity (www.genecards.org). Since IFNG gene has very high expression levels in this studied case, and it sustains IL12B expression and activity, it might be possible that IL12B to be also overexpressed. According to the functional interaction network in Figure 3, IL12A groups with IFNG.

TGFβ1 inhibits the development of T CD4+ lymphocytes, which produce IFNG, when antigen-presenting cells are lacking (Lin et al., 2005). According to Figure 3, TGFβ1 and IFNG are in separate groups, showing that their functional affinities are low.

IFNG stimulates CXCL10 activity (according to a not shown String® network, with required confidence of 0.7, and 100 interactors shown). CXCL10 has a chemoattraction activity on the regulatory T-cells (Rainczuk et al., 2012), which produces IFNG, a fact attested also by the increased expression of IFNG gene, by comparison with that of CXCL10 gene. CXCL10 has angiostatic (Balestrieri et al., 2008), (Sato et al., 2007) and anti-tumoral activity (Rainczuk et al., 2012). Both of the corresponding genes have very high levels of expression in tumor tissue.

Two overexpressed proteins, TNFSF15 and BAI1 appear to be disconnected from the main interaction network. Searcing all the possible interactions of these proteins with the main functional interactions network, we lowered the score (required confidence) to 1.5; in this situation, only TNFSF15 seems to interact with IFNG, whereas BAI1 still remains disconnected. Furthermore, adding 20 interactors, it appears that TNFSF15 interacts with IFNG, FIGF2, VEGFA, KDR (TNFSF15 has an inhibitory activity on endothelial cells, probably by inhibiting VEGFA and/or KDR activities), IL8, FLT1, CXCR3, CXCL10, while BAI1 seems to interact with FIGF2, VEGFA, ANGPT2 and HIF1A. By introducing only BAI1 protein and allowing the program to choose among 10 and 400 the most suitable interactors for it, using scores among 0.4 - 0.7, String® has shown that BAI1 expression is sustained by TP53, which appears often silenced (due to a mutational event) in tumors. This finding can give a clue that, in this patient, TP53 has its normal structure and function in the tumoral tissue. By introducing only TNFSF15 protein and allowing the program to choose 400 interactors for it, using the score of 0.4, it seems that IL13 sustains the TNFSF15 expression (networks not shown).

The very high expression levels of angiostatic factors CXCL10, IFNG and TNFSF15 can interrelate with the positive evolution of the patient after the resection of tumoral tissue. This also can interrelate with an effect of angiogenesis inhibition/reduction, due to the presence, in significant amounts, of angiostatic factors (Wicki, 2007), even if the main part of the analyzed angiogenic inducers appeared also overexpressed (Figure 4).

The case of this patient, from whom the tissue samples were prelevated, is one in which in appears that the angiostatic factors have a strong influence on angiogenic shift. It seems that not only the number of angiogenic and angiostatic factors is critical to the angiogenic balance tilting in favor of one or other process, but also the quantity of angiogenic and angiostatic factors, and their functional relationships (stimulation/inhibition) with other important factors involved in tumorigenesis.

The tumoral progression, with the occurrence of metastasis inside the ipsilaterocervical lymph node indicates a cell migration process through the blood- and lymphatic vessels; this event shows weak tumor immunogenicity. That is, the tumoral tissue fails to induce a T-helper cell response. However, this common situation for many tumor types leads to the release of the necessary citokines (in our case, IFNG, CXCL10, TNFSF15, IL12A, BAI1) that act as stimuli for the production of cytolitic T cells, which can fight against the spread of the
tumoral cells in other organs of the body, and can stop the tumoral evolution.

Emerging molecular therapies are focused also to increase the levels of the cytokines that stimulate activity of cytolytic T-cells, through infection with retroviruses carrying the genes for these proteins (Sikora, 2000).

**Fig. 2.** The functional interactions network among the VEGFA, VEGFB, VEGFC, IL8, PDGF, MMP2, TGB1, ANGPT2, FGF2, TIE2, CXCL12, IL12A, BA11, CXCL10, IFNG and TNFSF15 proteins, using the maximum required confidence level (0.9), 10 interactions added and all the available predictive methods.

**Fig. 3.** The protein functional interactions network from the Figure 1, built using MCL.

**Table 1.** The functional interaction types among the proteins with the 10 interactors added.

<table>
<thead>
<tr>
<th>Protein</th>
<th>VEGFA</th>
<th>VEGFB</th>
<th>VEGFC</th>
<th>IL8</th>
<th>PDGF</th>
<th>MMP2</th>
<th>TGB1</th>
<th>ANGPT2</th>
<th>FGF2</th>
<th>TIE2</th>
<th>CXCL12</th>
<th>BA11</th>
<th>CXCL10</th>
<th>IFNG</th>
<th>TNFSF15</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGFA</td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEGFB</td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEGFC</td>
<td></td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL8</td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDGF</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TGB1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANGPT2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FGF2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TIE2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CXCL12</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BA11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CXCL10</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFNG</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>TNFSF15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
</tr>
</tbody>
</table>

**Fig. 4.** The angiogenic balance in the analyzed tissue samples, shows that overexpressed angiostatic factors overcome the angiogenic factors.

**Conclusions**

In this study, we propose an original approach for the analysis of tumorigenic factors expressed in a specific tumor: a theoretical framework of angiogenic and angiostatic protein interactions, in order to find the way in which the type and amount of some proteins expression affect the expression and activity of other proteins involved in a tumoral process. Interesting, using such functional interaction networks, by analyzing
the expression pattern of some proteins, we can obtain information on the normal/abnormal function of other proteins which are functionally linked to the first ones (e.g. BAI1 expression is sustained by TP53 which appears often silenced in tumors, due to mutational events; thus, a high expression of BAI1 could reflect a normal status of TP53).

Using the protein functional interaction theoretical framework, the most significant gene products involved in a tumoral process can be identified for a specific tumor, and thus, more efficient therapeutic targets can be specified. Furthermore, the efficiency of the protein interactions shown in these networks can be verified in vitro and if they prove their validity, they can be tested later, in vivo. These frameworks can be used as a base for developing of some therapeutic strategies for tumor growth inhibition (i.e. improving of tumor immunity, oncogenes expression suppression, reactivation or replacing of damaged tumor suppressing genes etc.).

Abbreviations list: MMP2, matrix metalloproteinase 2 (gelatinase A, 72kDa gelatinase, 72kDa type IV collagenase); TGFB1, transforming growth factor, beta 1; IFNG, interferon, gamma; FGF2, fibroblast growth factor 2 (basic); VEGF, vascular endothelial growth factor C; IL12A, interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35); CXCL10, chemokine (C-X-C motif) ligand 10; IL8, interleukin 8; VEGFB, vascular endothelial growth factor B; BAI1, brain-specific angiogenesis inhibitor 1; ANGPT2, angiopeptin 2; PDGF, platelet-derived growth factor beta polypeptide; VEGFA, vascular endothelial growth factor A; TIE1, tyrosine kinase with immunoglobulin-like and EGF-like domains 1; TNFSF15, tumor necrosis factor (ligand) superfamily, member 15; CXCL12, chemokine (C-X-C motif) ligand 12; KDR, kinase insert domain receptor (a type III receptor tyrosine kinase); FLT4, fms-related tyrosine kinase 4; TGFBR2, transforming growth factor, beta receptor II; PDGFRB, platelet-derived growth factor receptor, beta polypeptide; NRP1, neuropilin 1.

Acknowledgements

The authors want to kindly thank to Acad. Octavian Popescu and to the research team from The Molecular Biology Center of the Institute of Interdisciplinary Researches in Bio-Nano-Sciences, from ‘Babes Bolyai’ University, Cluj Napoca, for providing the reagents needed to accomplish this study.

References

Nishimori H., Shiratsuchi T., Urano T., Kimura Y., Kiyono K., Tatsumi K., Yoshida S., Ono M., Kuwano M.,


Savage S.A., Abnet C.C., Mark S.D., Qiao Y.L., Dong Z.W., Dawsey S.M., Taylor P.R., Chanock S.J., Variants of the IL8 and IL8RB genes and risk for gastric cardia adenocarcinoma and esophageal squamous cell carcinoma, Cancer Epidemiol Biomarkers Prev.;13(12):2251-7, 2004;


Thurston G., Daly C., The complex role of angiopoietin-2 in the angiopoietin-tie signaling pathway, Cold Spring Harb Perspect Med.;2(9):a006550, 2012;


Witsch E., Sela M., Yarden Y., Roles for growth factors in cancer progression, Physiology (Bethesda); 25(2):85-101, 2010;

