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*ORIGINAL ARTICLE*

# Deregulation of EGFR/VEGF/HIF-1a signaling pathway in colon adenocarcinoma based on tissue microarrays analysis

D.N. Rigopoulos[1], E. Tsiambas[2], A.C. Lazaris[2], N. Kavantzas[2], I. Papazachariou[3], C. Kravvaritis[4], D Tsounis[5], A. Koliopoulou[6], A.E. Athanassiou[7], A. Karameris[8], L. Manaios[9], T.N. Sergentanis[2], E. Patsouris[2]

*[1]Department of Internal Medicine, 401 General Army Hospital, Athens; [2]Department of Pathology, Medical School, National and Kapodistrian University, Athens; [3]Department of Surgery, General Hospital Larnaca, Cyrpus; [4]Department of Forensic Service, Larissa; [5]Department of Gastroenterology, 251 Air Force Hospital, Athens; [6]Department of Thoracic Surgery, "Evangelismos" Hospital, Athens; [7]Department of Medical Oncology, "Metaxa" Cancer Hospital, Piraeus; [8]Department of Pathology, 417 VA-NIMTS Hospital, Athens; [9]Department of Surgery, "Bioclinic", Athens, Greece*

## Summary

***Purpose:*** *Overexpression of epidermal growth factor receptor (EGFR) and vascular endothelial growth factor (VEGF) in colon adenocarcinoma (CA) is a frequent event, whereas specific deregulation mechanisms in the corresponding signaling pathways remain under investigation. Our aim was to co-evaluate their expression correlated to the hypoxia inducible factor 1alpha (HIF-1a), which activates the transcription of VEGF gene.*

***Methods:*** *60 paraffin-embedded primary CAs were cored at 1.5 mm diameter and transferred to the microarray block. Immunohistochemistry (IHC) was performed using anti-EGFR, -VEGF, and -HIF 1a monoclonal antibodies. Concerning EGFR, quantitative evaluation was based on a semi-automated analysis system. Chromogenic in situ hybridization (CISH) was performed using EGFR gene and chromosome 7 centromeric probes.*

***Results:*** *Protein overexpression was observed in 13/60 (21.6%), 45/60 (75%) and 7/60 (11.6%) cases regarding EGFR, VEGF, and HIF 1a, respectively. CISH analysis detected 4/60 (6.6%) EGFR gene amplified cases, whereas chromosome 7 aneuploidy was identified in 11/60 (18.3%) cases. Significant associations raised correlating stage to chromosome 7 (p=0.024), HIF 1a expression to tumor anatomical location (p=0.019) and also VEGF to HIF 1a expression (p=0.001), whereas EGFR expression was not associated to EGFR gene copies.*

***Conclusion:*** *According to our results, chromosome 7 instability is correlated to advanced disease, whereas a significant subset of CAs demonstrates an alternative, non- HIF 1a depended mechanism of VEGF overexpression. Furthermore, EGFR protein overexpression does not predict a specific gene deregulation mechanism.*

**Key words:** colon adenocarcinoma, growth factors, hybridization, immunohistochemistry, microarrays

## Introduction

Deregulation of signal transduction pathways represents a crucial genetic event during carcinogenesis [1]. Concerning CA, which is one of the leading causes of cancer death in the western World, overexpression of growth factors seems to correlate with advanced disease [2,3]. EGFR gene is located on chromosome 7 (7p12) and its product is a 170 kDa protein, comprising 3 major functional domains: an extracellular ligand-binding, a hydrophobic transmembrane and a cytoplasmic tyrosine-kinase domain [4]. Ligands, such as EGF or TGF-α, bind to the extracellular domain of the receptor and trigger a cataract of reactions, including dimerization and phosphorylation of the intracellular part and finally signal transduction to nucleus is mediated by the involvement of RAS/RAF/MAPK proteins predominantly and via an alternative pathway (PI3/AKT/

*Correspondence to:* Evangelos Tsiambas, MD, PhD. Patriarchou Grigoriou E 17, 153 41 Ag. Paraskevi, Attiki, Greece. Fax: +30 210 7288249, E-mail: tsiambasecyto@yahoo.gr

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mTOR). In aggressive tumors, such as glioblastomas, EGFR gene amplification is correlated with shorter survival and resistance to radiotherapy [5]. Almost recently, novel targeted therapeutic strategies including anti-EGFR agents, such as monoclonal antibodies and small molecules have been approved for the treatment in EGFR-dependent cancers, including cancers of the colon, lung, and pancreas [6,7]. Although EGFR protein overexpression is observed in different proportions (25-80%) of CAs, the crucial process for a successive therapeutic approach (response to chemotherapy and to novel targeted agents, survival benefits) remains the identification of specific gene deregulation mechanisms [8,9]. Some studies have already suggested that there is an association between EGFR gene amplification and specific point mutations at exons 18-21 with disease prognosis [10,11].

Additionally, VEGF acts as a key mediator of angiogenesis in cancers of different origins [12]. VEGF gene is a member of the PDGF/VEGF growth factor family and is located on chromosome 6 (6p12). Its protein product (VEGF A) is a glycosylated mitogen acting as an endothelial cell growth factor, promoter of cell migration, and inhibitor of apoptosis [13]. Normally VEGF cytokine induces endothelial proliferation and increases vascular permeability, whereas deregulation of its upstream regulators, such as HIF-1a, a transcription factor responsible for the regulation of oxygen homeostasis, leads to a tumor-associated angiogenesis by its overexpression [14]. HIF 1a gene is located on chromosome 14 (14q21-24) and under normal oxygen microenvironment von Hippel-Lindau (VHL) targets its protein product for rapid ubiquitination and degradation. In contrast, under hypoxic conditions, HIF 1α is activated through PI3 kinase - AKT and MAPK-ERK pathways, binding with its complementary factor HIF 1β to the promoters of genes that mediate glycolysis and angiogenesis, such as VEGF [15]. Aberrant secretion of VEGF due to hypoxia, activation of oncogenes, and even EGFR or an abnormal hormonal activity leads to an uncontrolled binding to specific receptors such as VEGFR-1 or VEGFR-2 [16,17]. A cataract of reactions including phosphorylation of intracellular tyrosine-kinase chains leads to tumor angiogenesis characterized by an abnormal structurally and functionally vasculature [18].

In the present study, we analysed EGFR, VEGF and HIF 1a at the protein level by IHC, and also EGFR gene and chromosome 7 at the DNA level by CISH in order to identify potential simultaneous deregulations in CA cancer cell subpopulations correlated with clinicopathological parameters.

## Methods

### *Study group*

For the purposes of our study, we used for 60 formalin-fixed and paraffin-embedded tissue samples of histologically confirmed CAs obtained by surgical resection between 2005 and 2007. The 417 VA Hospital-NIMTS ethics committee gave permission to use those tissues for research purposes. Oral informed consent was obtained from each patient and the study protocol conformed to the ethical guidelines of the "World Medical Association Declaration of Helsinki - Ethical Principles for Medical Research Involving Human Subjects" adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964, as revised in Tokyo 2004. Ten microscopically normal-appearing colon epithelia were used as control group for the analysis. All corresponding hematoxylin and eosin (H&E)-stained slides were reviewed by two pathologists for confirmation of diagnosis and classification according to World Health Organization (WHO) grading criteria for CA. Furthermore, staging was assessed using the TNM staging system. Clinicopathological data are demonstrated in Table 1.

### *Tissue microarray (TMA) construction*

Areas of interest were identified in H&E stained slides by a conventional microscope (Olympus BX-50, Melville, NY, USA). Selection of those areas was performed on the basis of tumor sufficiency, avoiding sites of necrosis or bleeding. Using ATA-100 apparatus (Chemicon International, Temecula, CA, USA), all of the source blocks were cored two times (in order to secure the presence of each case in the final blocks) and 1.5-mm diameter tissue cylindrical cores were transferred from each conventional donor block to the 3 recipient blocks. After 3 mm microtome sectioning and H&E staining the final constructed TMA blocks contained 92% cores of tissue cylindrical specimens. We observed microscopically that all examined cases were represented by at least one or two tissue spots (confirmation of the adequacy of the examined specimens) (Figure 1a).

### *Antibodies and probes*

Ready-to-use EGFR monoclonal mouse antibody (clone 31G7-Zymed/InVitrogen, San Francisco, USA, dilution 1:10) recognizing predominantly the extracellular domain of EGFR protein and not reacting with

**Table 1.** Clinicopathological data and IHC results on 60 patients

| | *HIF-1α* | | | *VEGF* | | | *EGFR* | | |
|---|---|---|---|---|---|---|---|---|---|
| | *LL (n=53) %* | *OE (n=7) %* | *p-value* | *LL (n=15) %* | *OE (n=45) %* | *p-value* | *LL (n=47) %* | *OE (n=13) %* | *p-value* |
| Gender | | | NS | | | NS | | | 0.009[W] |
| Female (n=32) | 87.5 | 12.5 | | 28.1 | 71.9 | | 90.6 | 9.4 | |
| Male (n=28) | 89.3 | 10.7 | | 21.4 | 78.6 | | 64.3 | 35.7 | |
| Tumor diameter (cm) | | | NS | | | NS | | | NS |
| 0-3 (n=15) | 93.3 | 6.7 | | 20.0 | 80.0 | | 66.7 | 33.3 | |
| 4-6 (n=30) | 86.7 | 13.3 | | 23.3 | 76.7 | | 76.7 | 23.3 | |
| ≥7 (n=15) | 86.7 | 13.3 | | 33.3 | 66.7 | | 93.3 | 6.7 | |
| Grade | | | NS | | | NS | | | NS |
| I (n=22) | 91.01 | 9.09 | | 22.3 | 72.7 | | 77.3 | 22.7 | |
| II (n=31) | 87.1 | 12.9 | | 25.9 | 74.1 | | 81.7 | 19.3 | |
| III (n=7) | 85.8 | 14.2 | | 14.3 | 85.7 | | 71.5 | 28.5 | |
| TNM stage | | | NS | | | NS | | | NS |
| I (n=8) | 87.5 | 12.5 | | 25.0 | 75.0 | | 87.5 | 12.5 | |
| II (n=26) | 84.6 | 15.4 | | 19.2 | 80.8 | | 84.6 | 15.4 | |
| III/IV (n=26) | 92.3 | 7.7 | | 30.8 | 69.2 | | 69.2 | 30.8 | |
| Location | | | 0.019[W] | | | 0.098[S] | | | NS |
| Other than rectum-sigmoid (n=24) | 83.3 | 16.7 | | 12.5 | 87.5 | | 83.3 | 16.7 | |
| Rectum-sigmoid (n=36) | 91.7 | 8.3 | | 33.3 | 66.7 | | 75.0 | 25.0 | |

LL: loss or low expression (0, 1+), OE: overexpression (2+,3+)/ High: 76-97, Moderate: 109-121 (according to staining intensity levels detected by image analysis), NS: not significant, S: p-value derived from Spearman's rank correlation coefficient, W: p-value derived from Mann-Whitney-Wilcoxon test for independent samples. For other abbreviations see text

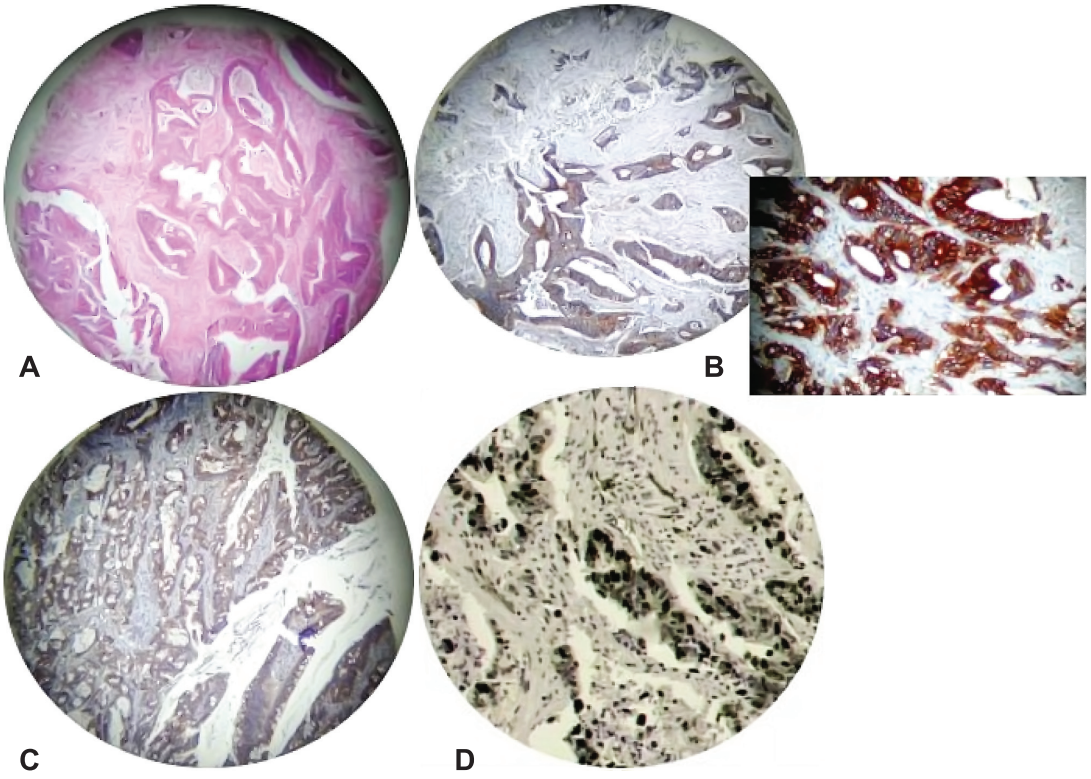


**Figure 1.** Tissue microarray cores of colon adenocarcinoma (CA) **A:** a case of moderately differentiated CA (H&E ×10); **B-D** simultaneous overexpression of the examined markers (EGFR, VEGF, HIF 1a, respectively). Original magnification x 10. Inset: Note EGFR membranous "ring-like", dense staining pattern (extended field ×40).

other erbB receptors was applied for the identification of protein expression. Furthermore, anti-VEGF (clone VG1-Diagnostic Biosystems, CA, USA) at dilution of 1: 40 and also anti-HIF 1a (clone H1alpha67-Thermo-Scientific/Neo markers, Fremont, CA, USA) at dilution of 1:30 were applied in the corresponding tissue microarray sections.

EGFR gene status was determined using the ready-to-use SPOT LIGHT EGFR DNA Probe (Zymed/InVitrogen, San Francisco, USA). This digoxygenin-labeled probe is located on 7p12 and covers the entire EGFR gene area. Similarly, chromosome 7 status was determined by the ready-to-use biotin-labeled chromosome 7 centromeric probe (Zymed/InVitrogen, San Francisco, USA) recognizing the specific repetitive centromeric DNA sequences known as α-satellite DNA.

*Immunohistochemistry (IHC) assay*

IHC for EGFR, VEGF and HIF 1a antigens was carried out on 3 μm serial tissue microarray sections. The slides were deparaffinized and rehydrated. Concerning EGFR, enzyme digested (proteinase K) for 10 min at 37º C was performed. The NBA kit (Zymed/InVitrogen, San Francisco, USA) was used. Blocking solution was applied to all of the slides for 10 min, followed by incubation for 1 h using the corresponding monoclonal antibodies at room temperature. Following incubation with the secondary antibody for 10 min, diaminobenzidine-tetrahydrochloride-DAB (0.03%) containing 0.1% hydrogen peroxide was applied as a chromogen and incubated for 5 min. Sections were counterstained, dehydrated and cover-slipped. For negative control slides, the primary antibodies were omitted. IHC protocol was performed by the use of an automated staining system (I 6000 - Biogenex, San Ramon, CA, USA). Membranous predominantly and sub-membranous cytoplasmic staining was considered acceptable for EGFR expression, diffuse cytoplasmic and membranous staining for VEGF, and nuclear/perinuclear staining pattern for HIF 1a according to manufacturers' data sheets (Figure 1b-d). Colon cancer tissue sections overexpressing EGFR and normal-appearing colon epithelia were used as positive and negative control, respectively. At first, EGFR protein expression levels were evaluated semi-quantitatively by two independent pathologists, using Zymed's Evaluation Guidelines. According to the scoring guidelines, the examined cases were classified as follows: score 0: no staining or membrane staining in <10% of tumor cells; score 1+: faint membrane staining in >10% of tumor cells; score 2+: weak or moderate complete membrane staining in >10% of tumor cells; and score 3+: strong, complete membrane staining in >10% of tumor cells. Scores of 0 and 1+ were considered as negative for EGFR expression while Scores 2+ and 3+ as positive for overexpression. This process was modified and performed for the evaluation of VEGF and HIF 1a, respectively.

*Chromogenic in situ hybridization (CISH) assay*

CISH SPOT-Light Chromogenic ISH Detection Kit was applied. CISH for chromosome 7 status and EGFR gene analysis was performed on 5 μm thick paraffin serial sections of the TMA block described above. Two slides were incubated at 37° C overnight followed by 2 h incubation at 60° C and then deparaffinized in xylene twice, 5 min each and in ethanol 3 times, 3 min each. The slides were rinsed in deionised water and then placed in a coplin jar containing CISH FFPE Pre-treatment Buffer (CISH Tissue Pre-treatment Kit, Zymed). For heat pre-treatment, the coplin jar was capped, loosely screwed, placed in a pressure cooker and timed for 10 min after the pressure built up. Then the slides were immediately washed in deionised water followed by enzyme digestion, which was performed by covering the sections with pepsin (CISH Tissue Pre-treatment Kit, Zymed) for 5 min at 37° C. The slides were washed with deionised water, dehydrated with graded ethanol and air-dried. Ready to use dig-labeled EGFR gene and biotin-labeled chromosome 7 centromere probes were applied to each section, respectively. Twenty microliters of probe were applied to each TMA section. Those tissue sections containing the added probe were denatured by placing the slides in a polymerase chain reaction (PCR) machine equipped with a slide block at 94° C for 5 min. The slides were then placed in a moist slide box and incubated at 37° C for overnight hybridization. The sections were stringently washed in 0.5× standard saline citrate at 75° C for 5 min. The CISH Polymer and the Horseradish (HRP) Detection Kit (Zymed/InVitrogen, San Francisco, USA) - containing similar steps to IHC - were used. Shortly afterwards TMA sections were placed in 3% $H_2O_2$ and diluted with methanol for 10 min to block endogenous peroxidase. To block unspecific staining, Cas Block$^{TM}$ (Zymed/InVitrogen, San Francisco, USA) was applied and incubated for 10 min. Following incubation with mouse anti-dig for 30 min and then polymerised HRP conjugated anti-mouse for 30 min, the EGFR probe was visualized by DAB development (CISH Polymer Detection Kit, Zymed). The biotin labeled chromosome 7 centromere probe was detected by incubation with HRP conjugated streptavid-

in for 30 min, followed by DAB development (CISH Centromere Detection Kit, Zymed) for 30 min. TMA sections were lightly counterstained with hematoxylin and dehydrated in graded ethanol. At the end of the process, CISH centromere signals or gene copies were easily visualized as dark brown/blue scattered or in small clusters dots, using a conventional bright-field microscope (Figure 1b). Interpretation of EGFR gene and chromosome 7 centromere signal results was based on Zymed's Evaluation Chart for CISH. According to this guide, two gene copies per nucleus demonstrate normal EGFR gene pattern, whereas 6-10 or small clusters characterize a low-level gene amplification. In this case, chromosome 7 status must be evaluated to exclude aneuploidy/polysomy (3-5 centromeric signals per nucleus; diploid pattern demonstrates normal chromosome status). High gene amplification level is characterized by the presence of more than 10 gene copies or large clusters of them per nucleus in more than 50% of the examined cells, whereas the presence of a smaller number of EGFR copies than chromosome 7 centromeric signals is considered to be an evidence of gene deletion (Figure 2).

*Computerized image analysis (CIA) assay*

In order to evaluate the IHC results-specifically 2+ and 3+ semi-quantitatively characterized cases regarding EGFR- in an accurate and faster way, we performed CIA by using a semi-automated system with the following hardware features: Intel Pentium Dual-Core, MATROX II Card Frame Grabber, Digital Camera Sony Cyber-shot, Microscope Olympus BX-50 and the following software: Windows XP/Image Pro Plus, version 5-Media Cybernetics 1997. Measurements of EGFR staining intensity values were performed in 5 optical fields per case at a magnification of 400× (Figure 3). All measurements were performed inside an active window of 16,848 $\mu m^2$. All numerical data were filed on Microsoft Excel sheets. Interpretation of staining intensity values (range 0-255) is demonstrated in Table 1.

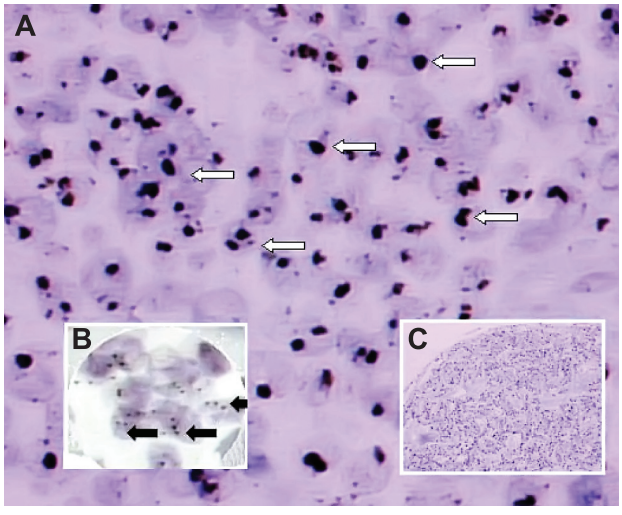


**Figure 2.** Numerical alterations detected by CISH. **A:** Note clusters of EGFR gene copies per nucleus (white arrows, high gene amplification, original magnification ×40). **B:** Note 3-5 chromosome 7 centromeric copies per nucleus (black arrows, aneuploidy, original magnification ×40). **C:** An arc of a tissue microarray core analysed by CISH (original magnification ×10).

*Statistical analysis*

Associations between variables including protein expression levels, gene and chromosome 7 alterations and clinicopathological parameters were performed by the application of STATA 8.0 statistical software (Stata Corporation, College Station, TX, USA). In the analysis, HIF-1α, VEGF, and EGFR, expression were treated as ordinal variables (0-3 scale); in the tables, however, the 0-1 (Loss or Low expression, "LL") and 2-3 /High-Moderate by image analysis for EGFR (overexpression, "OE") categories have been merged for reasons of brevity. Concerning the associations between these variables and other ordinal or continuous variables, p-value was derived form Spearman's rank correlation coefficient. For the evaluation of their associations with binary variables, Mann-Whitney-Wilcoxon test for independent samples was performed. Regarding the associations involving EGFR gene and chromosome 7 status (binary variables), Fisher's exact test, chi-square test / chi-square test for trend and Mann-Whitney-Wilcoxon test were appropriately performed. Total IHC and CISH results and also p-values are described in Tables 1-3.

## Results

IHC results were successfully obtained from all CA cases. EGFR overexpression was observed in 13/60 (21.6%) cases, whereas the rest of them (n=47) were characterized by low or negative protein expression. According to the conventional evaluation criteria, 7 cases were evaluated as 3+ and 6 as 2+. CIA for EGFR protein staining intensity levels showed that 5 cases demonstrated high values, and 8 cases moderate values. EGFR protein expression was statistically associated with gender ($p = 0.009$). VEGF overexpression was assessed in 45/60 (75%) cases and demonstrated no correlation with the examined clinicopathological parameters. HIF 1a was also overexpressed in 7/60 (11.6%) cases correlated with the anatomic location

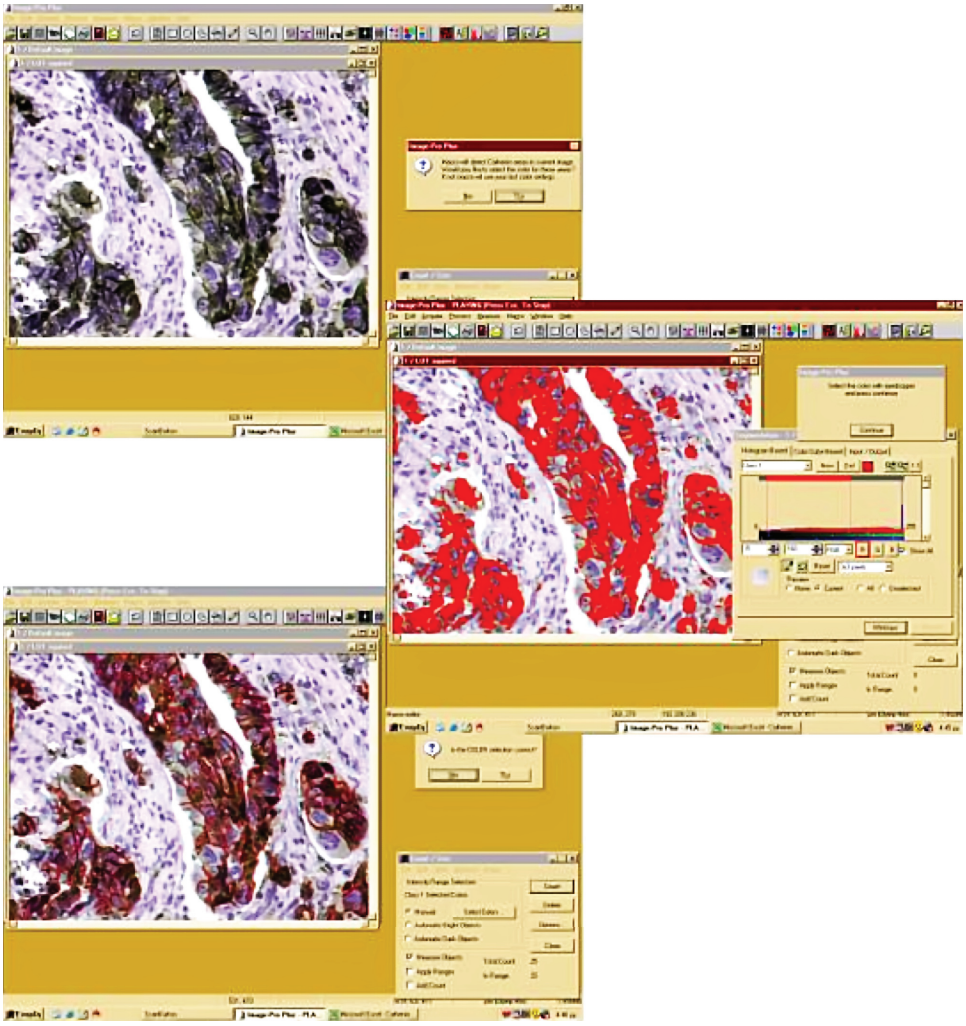

**Figure 3.** Computerized image analysis of EGFR protein expression levels. Reddish areas represent the membranous/submembranous cytoplasmic staining pattern (original magnification ×40).

**Table 2.** CISH molecular analysis results on 60 patients

| | *EGFR gene* | | | *Chr 7* | | |
|---|---|---|---|---|---|---|
| | *non-amplified (n=56)* % | *amplified (n=4)* % | *p-value* | *diploid (n=49)* % | *polysomy (n=11)* % | *p-value* |
| Gender | | | NS | | | NS |
| Female (n=32) | 96.9 | 3.1 | | 84.4 | 15.6 | |
| Male (n=28) | 89.3 | 10.7 | | 78.6 | 21.4 | |
| Age | 71.1±11.0 | 67.8±7.1 | NS | 70.5±10.8 | 72.8±11.1 | NS |
| Tumor diameter (cm) | | | NS | | | NS |
| 0-3 (n=15) | 86.7 | 13.3 | | 73.3 | 26.7 | |
| 4-6 (n=30) | 96.7 | 3.3 | | 86.7 | 13.3 | |
| ≥7 (n=15) | 93.3 | 6.7 | | 80.0 | 20.0 | |
| TNM stage | | | NS | | | 0.024[C] |
| I (n=8) | 87.5 | 12.5 | 7.7 | 100.0 | 0.0 | |
| II (n=26) | 92.3 | | | 88.5 | 11.5 | |
| III/IV (n=26) | 96.2 | 3.8 | | 69.2 | 30.8 | |
| Anatomic location | | | NS | | | NS |
| Other than rectum-sigmoid (n=24) | 87.5 | 12.5 | | 87.5 | 12.5 | |
| Rectum-sigmoid (n=36) | 97.2 | 2.8 | | 77.8 | 22.2 | |

non-amplified: normal (2 gene/centromeric signals per nucleus), gains due to polysomy; C: p-value derived from chi-square test for trend, Chr 7: chromosome 7. None of the examined parameters was associated with tumor grade; consequently, tumor grade has not been included in the Table

**Table 3.** Correlations between the evaluated markers

| *Marker* | *HIF-1a* | *VEGF* | *EGFR* | *EGFR gene* | *Chr 7* |
|---|---|---|---|---|---|
| HIF-1a | – | | | | |
| VEGF | (p=0.001) | – | | | |
| EGFR | NS | NS | – | | |
| EGFR gene | NS | NS | NS | – | |
| Chr 7 | NS | NS | NS | NS | – |

p-values derived from Spearman's rank correlation coefficient, Chr7: chromosome 7, NS: not significant. For other abbreviations see text

of the analysed tumors (p=0.019), while VEGF demonstrated a borderline correlation (p=0.098). Finally, statistical significance was determined by associating overall VEGF and HIF 1a expression (p=0.001). Interestingly, all the HIF 1a overexpressed cases demonstrated VEGF strong expression, but not *vice versa*. Furthermore, only one case of EGFR overexpression demonstrated also HIF 1a overexpression.

CISH results were also successfully obtained from all the examined cases based on tissue cores. EGFR gene was observed to be normal (2 gene copies predominantly per nucleus in tissue cores), in 46 cases, whereas the rest of the analyzed cases (n=14) demonstrated numerical alterations. In 13 of them, 3-10 gene copies per nucleus were identified, whereas in the last case, small to moderate clusters (accumulations of gene copies) were detected, evidence of high gene amplification. Chromosome 7 instability (polysomy, observed as 3-5 dots per nucleus) was detected in 11/60 (34.2%) cases, whereas the rest of them (49/60; 63.8%) demonstrated a normal, diploid pattern. Correlating EGFR gene copies to chromosome 7 centromeric signals in the 13 previous referred cases that demonstrated 3-10 gene copies per nucleus, we observed that 10 of them were characterized by polysomy and only the rest of them (n=3) were purely amplified. Significant statistical correlation was observed only by associating chromosome 7 instability to stage (p=0.024), whereas we did not observe a statistical significance correlating EGFR gene status with the examined clinicopathological parameters.

## Discussion

Although advances in the treatment of CA (new surgical resection modifications combined with adjuvant chemotherapy) have been achieved, novel targeted therapeutic strategies require knowledge of specific deregulation mechanisms regarding signaling transduction pathways in order to be effective (response to therapy, survival benefits) [19]. Some recently published studies support strongly the idea that EGFR protein overexpression alone is not the eligible criterion in selecting patients for administration of targeted therapeutic monoclonal antibodies due to its binding on the receptor's extracellular domain [20,21]. Furthermore, EGFR overexpression does not predict a specific gene deregulation mechanism in contrast to HER2/neu, another member of EGFR type I superfamily. Concerning breast cancer, the receptor's overexpression is correlated significantly to gene amplification [22,23]. This parallelism shows that EGFR inhibition strategies in CA must be based on a combination of the protein expression and the corresponding genetic deregulation (gene amplification/point mutations).

In the current basic research study, IHC analysis identified VEGF overexpression in the majority of the examined cancer cell subpopulations, whereas EGFR and HIF-1a molecules were overexpressed at a relatively low percentage. Many studies have demonstrated different percentages of EGFR overexpression in CA specimens and also in cancers of different origins [24-26]. This broad range of controversial results is easily explained from the technical point of view due to selection and application of different antibody clones and IHC protocols. Furthermore, differences in the fixation of the paraffin-embedded specimens and also the geographical origin of the examined patients, which potentially is correlated to specific genetic alterations regarding colon carcinogenesis, modifies the protein expression levels. Additionally, other agents such as the recently cloned EGFR-related protein and interferon alpha regulate the expression levels of EGFR and affect indirectly the IHC results [27, 28].

In contrast to the relativity, which characterizes IHC results, DNA analysis brings out the details in genetic imbalances. In our study, we performed a CISH protocol and identified EGFR gene and chromosome 7 numerical alterations. CISH is an alternative to conventional fluorescence *in situ* hybridization (FISH) technique characterized by many advantages. Numerical alterations are visualized under conventional bright field microscopy due to an IHC-like process using conventional chromogens (DAB) and can be permanently stored on the corresponding slides. Some recently published studies have concluded that CISH and FISH results demonstrate a high level of concordance (92-98%) in identifying HER2/neu or EGFR gene alterations [24,29**].** Although gene amplification was detected in a small subset of our examined cases, this mechanism seems to be associated with a more rational selection of patients for treatment with cetuximab, a recombinant anti-EGFR monoclonal antibody, and/or of panitumumab, a fully humanized one [10]. The majority of the overexpressed tumors were characterized by

a normal gene pattern. Mutations of the tyrosine kinase intracellular sequences or alterations at the mRNA level represent alternative causes for the protein overexpression [30]. Furthermore, chromosome 7 aneuploidy was identified in a significant subset of cancers and strongly correlated to their stage. Extensive genomic analysis has detected a variety of chromosomal abnormalities in CA involving predominantly chromosomes 13,17,8 and 7. Instability in specific gene loci (gains or losses) of them affects the biological behavior of CAs associated with an aggressive phenotype (advanced stage/grade, resistance to chemotherapy) [31].

Protein analysis of the VEGF/HIF 1a pathway showed that the majority of the examined cases overexpressed VEGF, whereas only a subset of them simultaneously expressed in a moderate or high-level the HIF 1a protein. Additionally, correlation of the overall expression of these two markers was found to be statistically significant. HIF 1a demonstrated also a strong association with the anatomical origin of the examined tumors. Interestingly, non rectum-sigmoid cancers overexpressed more frequently the protein than cancers of rectum-sigmoid origin. Differences at the biochemical microenvironment during the carcinogenetic process are responsible for these identified imbalances. It is known that not only HIF-1a hypoxia-dependent aberrant expression is responsible for VEGF overexpression but also there are alternative pathways, such as the HER2/neu- dependent PI3K/AKT/m TOR, which upregulates its expression [32]. Furthermore, another alternative mechanism of VEGF/HIF 1a pathway deregulation is associated to c-myc and also k-ras oncogenes [33,34]. It seems that there are multiple mechanisms including HIF 1a-dependent and HIF 1a-indepentent VEGF expression in CA. Interestingly, simultaneous deregulation of VEGF/HIF 1a molecules appears to be the eligible biochemical substrate for anti-angiogenetic targeted strategies based on monoclonal antibodies, such as bevacizumab and also on new HIF 1a inhibitors including agents such as 1a,25-dihydroxyvitamin (D3) and even caffeine [35-37]. In addition, VEGF overexpression detected by HIC correlates potentially to the tumors' stage, although there are controversial published results [38]. Concerning our results, the majority of VEGF overexpressing tumors demonstrated moderate to low levels of HIF 1a, an evidence of a HIF 1a-indepentent upregulation of VEGF protein. Additionally, statistical significance was not assessed correlating EGFR and HIF-1a overall expressions. Based on this observation we can speculate that specific downstream molecules of the EGFR-dependent signaling pathway are involved in the regulation of HIF -1a expression and function.

In the present study, we performed a combination of tissue microarrays and CIA in order to evaluate the EGFR protein expression using a rapid, accurate and quantitative method. Comparing the results of the conventional eye-microscopy evaluation and CIA we observed that some differences raised, especially in the characterisation of borderline (2+/3+) overexpression cases. Digital analysis detected specific staining intensity levels in contrast to human eye, which is characterized by limitations in discriminating those slight differences. Although the overall statistical analysis concerning EGFR expression was not dependent on the evaluation method, the cases of EGFR high gene amplification (>10 multiple signals and also clusters) demonstrated the highest protein staining intensity levels. This is an interesting observation reflecting the need for integrated methods in modern pathology, such as CIA procedures.

In conclusion, different subsets of patients with CA demonstrate specific protein and gene alterations regarding EGFR and VEGF/HIF 1a molecules. This event maybe the basis for more rational anti-angiogenetic targeted therapeutic strategies combined with the knowledge of the molecular deregulation mechanisms of these molecules (i.e. EGFR amplification) [39]. Furthermore, the study showed a strong correlation between chromosome 7 aneuploidy and advanced disease stage (increased p-stage).

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