THE PROGNOSTIC VALUE OF THE ANGIOGENIC MARKERS IMMUNOHISTOCHEMICALLY AND GENETIC INVESTIGATED IN COLORECTAL CANCER

ABSTRACT

SCIENTIFIC SUPERVISOR, Professor dr. MIHAI CRUCE
Ph student, COSMIN ȘTEFAN JALBĂ

CRAIOVA
2011
LIST OF ABBREVIATIONS

CRC – colorectal cancer
LVI – limfovascularar invasion
MVD – microvascular density
OS – overall survival
PFS – progression free survival
SNP – single nucleotid polymorphism
VEGF – vascular endothelial growth factor
INTRODUCTION

Over 100 years ago it was observed that tumoral tissue had an increased vascularity compared to normal tissue. In the last 50 years the therapeutic approach in cancer has been the direct action on the tumor cells. Administration of cytotoxic drugs that kill tumor cells \textit{in vitro} has been the method of choice for chemotherapy \textit{in vivo}.

It was found however, that along with tumor cells, normal cells might be also affected by cytotoxic drugs. In addition, due to the genetic instability of these tumor cells, chemotherapy does not always have the expected results.

The anti-angiogenic tumoral therapy concept has its origins in the observations of Folkman (1995) - an increase of the tumoral mass to more than 2-3 mm$^3$ depends on the novo formation of a new vascular network capable of supplying the tumor with oxygen and nutrients. This assumption, now proven by a large number of experimental studies, suggests that a tumor can be stopped (or destroyed) by inhibiting the development of their vasculature. In this context, the \textit{main objectives of the thesis} are:

- to evaluate the prognostic significance of MVD in tumour tissues and the survival rate in patients with colorectal cancer;
- to study the expression status and clinical relevance of vascular endothelial growth factor-A (VEGF-A) in colorectal cancer (CRC) tissues.

- to assess the significance of angiogenesis by VEGF expression in patients with colorectal cancer in order to determine the individuals with a high degree of recurrence;

- to analyze VEGF gene polymorphisms and their effect on the prognosis for patients with colorectal cancer;

- to analyse the prognostic influence of VEGF-A gene haplotypes in patients with stage II or III colorectal cancer;

- to determine the proliferative activity of tumor cells with the Ki67 and p53 antibodies and with VEGF, CD31 and CD105 in order to quantify angiogenesis.

**Keywords**: colorectal cancer, tumor angiogenesis, tumor microvascular density, VEGF-A polymorphisms, haplotype.

**I. REVIEW OF THE PRESENT KNOWLEDGE**

Chapter 1 entitled "General aspects of tumor angiogenesis” analyzes the assumptions on the dependence of the tumor growth on angiogenesis, tumor angiogenesis relations with the metastasis, angiogenic and antiangiogenic factors and the mechanisms of tumor angiogenesis and tumor development.
Chapter 2 - entitled "Prognostic factors in colorectal cancer" analyzes the prognostic factors related to the primary tumor, the clinical factors with prognostic significance and the histopathologic prognostic factors.

II. PERSONAL CONTRIBUTIONS

Chapter 3 – “Prognostic significance of intratumoral microvascular density in patients with colorectal cancer” successively presents materials and methods, results and discussions on the immunohistochemical marker CD105 and the pan-endothelial marker CD31, as well as on the correlations between a high microvascular density and the survival rate in colorectal cancer, techniques that I performed on 120 samples tissues from patients with colorectal cancer and who underwent surgical resection of the tumor in the Emergency Hospital "Floreasca" in Bucharest.

Kaplan–Meier survival analysis indicated that MVD obtained using CD31 showed no significant correlation with survival. The same result was noted when MVD was divided by either median value or by quartiles, demonstrating that microvessel counts using CD31 were not correlated with prognosis. In sharp contrast, high MVD counts determined using CD105 were strongly associated with a poor prognosis (Figure 1).
Fig 1. Kaplan–Meier survival graphs showing percent survival of patients with CRC. MVD data obtained using CD31 and CD105 were divided into above and below median (A, C) and into quartiles (B, D). Microvessel density values obtained using CD105 showed that high microvessel counts indicated poor prognosis, but there was no significant correlation with MVD values given by CD31. In addition, patients with Dukes’ stage C and D (E) or positive lymph nodes (F) survived the shortest time. The P-values were obtained by log-rank tests.
As it is shown in figure 2, CD31 and CD105 staining was heterogeneous. Areas most heavily vascularized were invariably observed at the periphery of the tumor and nearby normal tissues.

Figure 2 Expression of CD105 and CD31 in tumour vasculature. Serial sections of colon carcinoma were stained with
CD105 or CD31. Although the majority of blood vessels were stained by both, a proportion of the vessels reacted only with CD105 but not with CD31, demonstrating the distinctive expression of CD105 and CD31 in tumour vasculature (x250).

That the MVD using CD31 was not correlated with prognosis raises important issues. It is not the first time that determination of MVD using a pan-endothelial marker has failed to be correlated with prognosis.

The conclusions are that a high MVD, identified using CD105, predicts a poor prognosis, and that circulating CD105, levels are positively correlated with Dukes’ stage. Therefore, CD105, as a novel marker of tumour angiogenic activity, may prove to be valuable in assessing the prognosis of patients with colorectal cancer, especially in those patients who are receiving antiangiogenic therapies.

Chapter 4 presents the clinical relevance of VEGF-A expression in colorectal cancer, immonohistochemical study on 89 samples taken from patients with operated CRC.

The most important finding of this study is that the survival was shorter in cases with strong VEGF-A expression, and that the differences were very evident according to the level of expression of VEGF-A (Table 1).
<table>
<thead>
<tr>
<th>VEGF-A</th>
<th>stage II</th>
<th>Stage III</th>
<th>Stage IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>(-)</td>
<td>75/-</td>
<td>30/12</td>
<td>6/6</td>
</tr>
<tr>
<td>(+)</td>
<td>62/48</td>
<td>26/24</td>
<td>36/6</td>
</tr>
<tr>
<td>(++)</td>
<td>50/48</td>
<td>21/24</td>
<td>11/6</td>
</tr>
<tr>
<td>(+++)</td>
<td>11/6</td>
<td>11/12</td>
<td>6/6</td>
</tr>
<tr>
<td>Death/total</td>
<td>18/40</td>
<td>22/26</td>
<td>9/11</td>
</tr>
<tr>
<td>P value</td>
<td>0,3</td>
<td>0,07</td>
<td>0,5</td>
</tr>
</tbody>
</table>

There was an important association of involved lymph node number with tumor stage and tumor relapse. Although the correlation was not statistically significant, there was a trend for higher grade of tumors to exhibit more LVI potential and more distant metastasis. Higher number of involved lymph nodes was also associated with higher tumor grade, higher metastatic potential and more SI.

According to these findings, VEGF-A may be used to identify the patients at high risk of relapse who may benefit from adjuvant strategies such as chemotherapy and/or antiangiogenic treatment. In our study, there were 48 cases with the stage II disease and two thirds of these showed VEGF-A expression. As suggested by some other studies, VEGF-A may be predictive to
determine the population requiring adjuvant chemotherapy in stage II disease.

Chapter 5 – VEGF-A gene polymorphisms and the association with prognosis in patients with CLC cancer.

Four hundred and forty-five patients with surgically treated colorectal adenocarcinoma were enrolled in the present study. The genomic DNA was extracted from fresh colorectal tissue and three VEGF (-2578C>A, -634G>C, and +936C>T) gene polymorphisms were determined using a PCR/denaturing high-performance liquid chromatography assay.

Multivariate survival analysis showed that the survival for the patients with the -634 G/C genotype or C/C genotype were better than for the patients with the -634G/G genotype, whereas the +936 C/T genotype or T/T genotype was associated with a worse survival compared with the +936 C/C genotype. In the haplotype analysis, the -2578A/-634G/+936T haplotype exhibited a significantly worse survival when compared with the wild-2578C/-634G/+936C haplotype.

Capitolul 6 is entitled “The prognostic value of VEGF-A gene haplotype in colorectal cancer”.

The objective of the present study was to investigate the prognostic importance of haplotypes in the VEGF-A gene in patients with CRC. The study included 486 patients surgically resected for stage II and III CRC, divided into two independent cohorts. Three
SNPs in the \textit{VEGF-A} gene were analyzed by polymerase chain reaction.

The $-2578$ C/A and the $405$ G/C SNPs both demonstrated prognostic value independent of the standard prognostic markers regarding DFS. A possible independent value, although not significant, was seen for the $-460$ C/T SNP. A similar multivariate survival analysis adjusted for the same variables was initially performed on the test cohort, with similar findings although the $405$ G/C SNP failed to demonstrate an independent prognostic value.

The $-2578$ C/A, the $-460$ C/T and the $405$ G/C SNPs all showed a significant relationship with survival, and in all three cases the heterozygous genotypes were related to poor survival.

\textbf{Chapter 7 – General conclusions}

The conclusions are that a high MVD, identified using Mab to CD105, predicts a poor prognosis, and that CD105 is positively correlated with Dukes’ stage. Therefore, CD105, as a novel marker of tumour angiogenic activity, may prove to be valuable in assessing the prognosis of patients with colorectal cancer, especially in those patients who are receiving antiangiogenic therapies.

Anti-angiogenic treatment is a novel approach in various malignant tumors. Targeting the tumor vasculature has been found to be useful both in vitro and in vivo studies. CRC is an important model to target the VEGF-A. It is known that the
antibody targeting the VEGF-A (bevacizumab) and small molecule inhibitors specific for the receptor tyrosine kinase for VEGF (SU5416 and ZD6474) are the important drugs for tailored treatment in clinical setting. For these reasons, to determine the tumor angiogenesis by VEGF or other parameters will be informative for tumor biology/prognosis and also tailored treatment in cases with CRC as seen in other tumors.

VEGF gene polymorphisms were found to be an independent prognostic marker for patients with colorectal cancer. Accordingly, the analysis of VEGF gene polymorphisms can help identify patient subgroups at high risk of a poor disease outcome.

In conclusion, this validating study of nearly 500 patients from two independent cohorts with stage II and III CRC identified a genetic signature related to the prognosis of patients with stage II and III CRC based on genetic variations in the promoter and 5’UTR of the VEGF-A gene. Analysing haplotype combinations, we were able to identify a group with a rather favorable prognosis and a group in which adjuvant chemotherapy seems indicated. The possible benefit from such a treatment, however, cannot be assessed from the present results. This unfavorable haplotype combination remained an independent prognostic marker. Future studies should focus more on haplotype analyses because of higher degrees of consistency between studies. The haplotype combination approach calls for further investigation.
SELECTIVE BIBLIOGRAPHY


CURRICULUM VITAE

Name: JALBĂ
Surname: COSMIN-STEFAN
Date of birth, City: 23.09.1979, CRAIOVA
Citizenship: Romanian
Civil status: married

Studies:

Professional experience:
UMF Craiova october 2007 - now – PhD student.
Sanador Medical Center – 2008 – now.

Foreign languages: English, German

Papers published

I. Papers published ISI:
II. Papers published CNCSIS:


III. Scientific papers presented


