C-ABL GENE EXPRESSION IN GASTRIC CANCER

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Summary

The aim of the study was to observe the differences between the expression of c-abl inside the tumor and at the resection limit in patients with gastric cancer. We use for our study 20 patients with gastric cancer with ages between 49 and 79 years with a median of 65 years, with a repartition on sexes 12 men and 8 women. We collected after the surgical intervention two samples of tissue: one sample from the tumor process and another sample from the gastric apparent normal tissue, as far from the tumor as it was possible. Quantitative real-time polymerase chain reaction was used to determine c-abl gene expression. Considering gastric cancer and the c-abl gene expression, c-abl is down regulated inside tumor cells comparing to the normal gastric tissue from de resection limit. This underlines the role of c-abl in normal tissue growth and its ability of inducing apoptosis when alteration of DNA occurs, as a result of different agents as stress, ionizing radiations. Down-regulation or loss of expression of c-abl is a fundamental event that leads at tumor apparition and progression.

Key words: c-Abl, gastric cancer, apoptosis, QRT-PCR.

Introduction

c-Abl is an enzyme that is involved in many cell processes, such as cell division. The gene for c-Abl is on chromosome 9. In most patients with chronic myelogenous leukemia (CML), the part of chromosome 9 with c-Abl has broken off and traded places with part of chromosome 22 to form the BCR-ABL fusion gene.

c-Abl is a ubiquitously expressed protein tyrosine kinase activated by DNA damage and implicated in two responses: cell cycle arrest and apoptosis. The downstream pathways by which c-Abl induces these responses remain unclear.

Cellular transformation, the conversion of normal cells into tumorigenic cells in vitro, is characterized by immortalization, anchorage- and serum-independent growth and tumor formation in the nude mouse.

Among these, anchorage-independent growth is one of the defining characteristics of transformed cells and tumor cells.

Without attachment to the extracellular substrate, most normal cells cannot grow or survive, but tumor cells can proliferate. Many oncogenes and tumour suppressors are involved in regulating this process, among which is Abl tyrosine kinases.

The cellular context, such as a deficiency in both p53 and retinoblastoma gene product, is critical to induce anchorage independence by loss of c-Abl kinase. Recent review, discuss the mechanisms of cellular transformation by oncogenic and normal Abl kinases (Suzuki and Tomoyuki, 2007).

Material and method

Sample prelevation

We use for our study 20 patients with gastric cancer, who underwent surgical intervention for this disease, having curative intention or for palliation, in II-nd
Surgery Clinic of Clinical Regional Emergency Hospital from Craiova. The age of the patients selected for this study was between 49 and 79 years with a median of 65 years, with a repartition on sexes 12 men and 8 women.

All collected tissues were examined at the laboratory of anatomo-pathology to establish the histological type, the degree of differentiation, and the tumor state. We described the localization of the neoplasm, as well as either the limit of resection is invaded or not. We collected after the surgical intervention two samples of tissue: one sample from the tumor process and we encoded it with A and another sample from the gastric apparent normal tissue, encoded with B as far from the tumor as it was possible. At the anatomo-pathological test we followed if resection limit presented tumor invasion or not.

The probes were collected inside a tube containing solution for RNA stabilization (RNA Later Solution, Ambion), then stocked at 4°C for 12-24 hours and finally transferred at -80°C. Every patient was informed and agreed to take part in this study. The written agreement was taken from each patient.

Isolation of total RNA

For total RNA isolation from collected probes we used Total RNA Isolation System SV kit (Promega, Madison, WI). This technique uses the guanidine thiocyanate (GTC) and the β-mercaptoethanol, which inactivate the ribonucleases present in cellular extracts. GTC combined with SDS (dodecil sodium sulfate) acts to destroy the nucleoproteic complexes, allowing the liberation of RNA in solution and its isolation, purified from proteins.

Dilution of cell extracts in the presence of high concentrations of GTC determines selective cellular protein precipitation, while RNA remains in solution. Following centrifugation to remove precipitated proteins and cellular debris, RNA is selectively precipitated in ethanol and bound to the silica surface of glass fibers in the filter basket.

After removing the protein precipitate and debris cell lysates is bound to filter basket by centrifugation. DN-ase I without RN-ase (RNase-free DNase I) is then applied directly on the membrane surface to digest contaminated genomic DNA. Total RNA bound is then purified by contaminating salts, proteins and cellular impurities by washing steps. Finally, total RNA is eluted from the membrane by adding water without nucleases. This procedure resulted in obtaining pure fractions of total RNA, whose concentration and purity was measured by spectrophotometry. To determine the quality of total RNA isolated, we analyzed the integrity of 18S and 28S ribosomal bands by denaturant agarose gel electrophoresis. Photographed gels were analyzed using analysis system G: BOX Chemi equipped with high resolution CCD camera.

Quantitative real-time polymerase chain reaction

Gene expression was analyzed by QRT-PCR reaction in two steps: reverse transcription followed by QRT-PCR. First, complementary DNA (cDNA) was synthesized from total RNA. In the second stage, PCR products quantitative are synthesize of cDNA.

A. Reverse transcription of total RNA to complementary DNA

For reverse transcription of total RNA to complementary DNA (cDNA) we have used the kit High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Reverse transcription reaction was performed in volumes of 20 μL / reaction 10 μL RT Master Mix 2X, and 10 μL total RNA. Cycling was performed with Eppendorf Thermocycler site to indicated protocol parameters of reverse transcription.
Table 1 - Protocol parameters temperature and time of reverse transcription

<table>
<thead>
<tr>
<th>Temperature</th>
<th>25°C</th>
<th>37°C</th>
<th>85°C</th>
<th>4°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time</td>
<td>10 min</td>
<td>120 min</td>
<td>5 min</td>
<td>∞</td>
</tr>
</tbody>
</table>

Evaluation of concentration and purity of cDNA was performed using Eppendorf spectrophotometer Biophotometer.

It was also performed reverse transcription standard RNA concentration 50 ng / μL product company Applied Biosystems, Foster City, CA. The concentration of cDNA obtained from RNA's standard was 325 mg / mL.

B. Polymerase chain reaction in real time (Real-Time PCR)

At this stage products PCR are synthesized from cDNA using TaqMan Gene Expression Master Mix and TaqMan probes Gene Expression assays (Applied Biosystems, Foster City, CA) specific for ABL1.

Real-Time PCR amplification

PCR reaction was carried out in volumes of 20 μL. cDNA samples were diluted in water without nucleases (Nuclease Free Water) at a concentration of 10 ng cDNA / μL. Final reaction mixture contained 80 ng cDNA (8 μL cDNA diluted). GAPDH was used as endogenous control of the reaction. We generated the standard curve for c-ABL using a standard RNA in order to quantify cDNA in patients’ samples. GAPDH was used as an internal control. Each sample was analyzed in triplicate. Cycling was performed by Real-Time System Corbet 6200 HRM RotorGene at the parameters presented in table below.

We also analyzed gene expression pattern using Genex pro 4.4.2.308©.

Table 2 – qRT-PCR Cycling

<table>
<thead>
<tr>
<th>Stage</th>
<th>UGD incubation</th>
<th>Activation AmpliTaqGold, UP</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Storage</td>
<td>Storage</td>
<td>Cycle (50 cycles)</td>
</tr>
<tr>
<td></td>
<td>Denaturation</td>
<td>Ataching/Extension</td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>2 min</td>
<td>10 min</td>
<td>15 sec</td>
</tr>
<tr>
<td>Temperature</td>
<td>50°C</td>
<td>95°C</td>
<td>95°C</td>
</tr>
</tbody>
</table>

Results and discussion

We aimed to observe the differences between the expression of c-abl inside the tumor and at the resection limit at patients with gastric cancer.

The results were generated with Real-Time PCR Rotor-Gene 6200 HRM software version 1.7 (Corbett). Gene expression levels were analyzed by using Microsoft®Office Excel® XP Professional; the values were logarithmated to base 2. We also analyzed gene expression pattern using Genex pro 4.4.2.308©.

Considering the gastric neoplasm we must define first the histological type inside the tumor. The relevance of the results has the histological diagnosis as a base. So, in our study, from the 20 patients, 17 patients presented gastric adenocarcinoma, 2 patients presented gastric adenocarcinoma mixed with neuroendocrin tumor and 1 patient presented gastric lymphoma with large cell.

Two of the patients with gastric adenocarcinoma had microscopic tumor invasion present at resection limits; so, the results are considered being non-relevant.
for these samples (no normal tissue to compare to).

We calculated and compared log2 from the value obtained from the tumor and respectively from the resection limit. The data obtained is shown in the chart 1 below.

From the 20 samples we obtained in 10 cases a lower gene expression for c-abl in tumor compared to the resection limit. In all these cases the variety of tumor was adenocarcinoma with different degrees of differentiation, from well differentiated to undifferentiated, having less or more local or lymphatic extent (samples 1, 3, 4, 5, 6, 7, 11, 12, 14, 17).

One case was gastric lymphoma (2) and the c-abl expression was low in tumor as well in the resection limit, with a higher level in the tumor tissue.

Two cases are considered irrelevant (8, 10) because of the microscopic tumor invasion of resection limits.

Two cases expressed mixed tumor cells inside – adenocarcinoma and neuroendocrin tumor (15, 20). In these cases the c-abl was over expressed inside neoplastic cells compared to the resection limit.

In 4 cases the c-abl expression has not shown significant differentiations between the tumor and resection limit. In these samples the histological type was adenocarcinoma.

Finally, one case of adenocarcinoma expressed a significant higher level for c-abl expression in the tumor and a lower expression in the resection limit with no microscopic invasion.

The Threshold line is the level of detection or the point at which a reaction reaches a fluorescent intensity above background. The threshold line is set in the exponential phase of the amplification for the most accurate reading. The cycle at which the sample reaches this level is called the Cycle Threshold.

Although, the Cycle Threshold (Ct) method is the present "gold standard", it is far from being a standard assay. Uniform reaction efficiency among samples is the most important assumption of this method.

In chart 2 we can easily observe that left columns are predominant higher, that means a lower gene expression of c-Abl inside tumor tissue. Our study observes a
lower level for c-abl expression inside gastric tumor tissue compared to the limit of resection.

From these data put together, we can conclude that, considering gastric cancer and the c-abl gene expression, c-abl is down regulated inside tumor cells comparing to the normal gastric tissue from de resection limit (with no microscopic tumor invasion). This underlines the role of c-abl in normal tissue growth and its ability of inducing apoptosis when alteration of DNA occurs, as a result of different agents as stress, ionizing radiations. Down-regulation or loss of expression of c-abl is a fundamental event that leads at tumor apparition and progression.

A striking feature of both wild-type c-Abl and the fraction of transforming c-Abl that is localized in the nucleus is their proliferation-suppressive effects (Sawyers et al., 1994; Wen et al., 1996). This may be accompanied by cytotoxic effects (Wen et al., 1996).

Abl proteins play important roles in cell homoeostasis, as determined by gene knock-out experiments in mice. abl ablation induces pleiotropic defects, which cause post-partum mortality, as well as lymphopenia and osteoporosis in the surviving animals. c-Abl shuttles between the nuclear and cytoplasmic compartments.

The role of nuclear c-Abl has been largely documented (Van Etten, 1999). Indeed, it modulates the cellular response induced by DNA damage, and has been implicated in cell growth inhibition and promotion of apoptosis. In contrast, the role of cytoplasmic c-Abl has not been thoroughly described. Genetic and biochemical analysis point to a central role for cytoplasmic c-Abl in morphogenesis and F-actin dynamics (Woodring et al., 2003).

The c-Abl protein tyrosine kinase localizes to the nucleus and cytoplasm. Nuclear c-Abl is activated in the response to DNA damage (Kharbanda et al., 1995)
by the DNA-dependent protein kinase (Jin et al., 1997) and the product of the gene mutated in ataxia telangiectasia (Baskaran et al., 1997). Activation of nuclear c-Abl by genotoxic stress contributes to induction of the proapoptotic c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 mitogen-activated protein kinase pathways (Kharbanda et al., 2000). Nuclear c-Abl also contributes to DNA damage-induced apoptosis by mechanisms in part dependent on the p53 tumor suppressor and its homolog p73 (Gong et al., 1999, Yuan et al., 1996 and 1999). Other studies have demonstrated that the cytoplasmic form of c-Abl is activated in the cellular response to oxidative stress (Sun et al., 2000). Reactive oxygen species induce cytoplasmic c-Abl activity by a mechanism dependent on protein kinase Cδ (PKCδ) (Sun et al., 2000).

c-Abl appears to have antagonistic function, depending on its subcellular localization. Accordingly, nuclear c-Abl can induce a G1-phase block of the cell cycle, whereas cytoplasmic c-Abl can promote mitogenesis (Vigneri and Wang, 2001). The positive mitogenic role of cytoplasmic c-Abl has been uncovered in abl-deficient cells. These cells exhibit a 4–6 h delay in DNA synthesis following PDGF and serum induction (Plattner et al., 1999; Furstoss et al., 2002). c-Abl has been then identified as an important effector of Src for mitogenic signaling (Furstoss et al., 2002), which is required for DNA synthesis.

c-Abl plays important functions in F-actin dynamics, yet its role in growth-factor-induced cell motility is not well defined. An inhibitory role was originally described to occur during cell adhesion and migration when induced by the extracellular matrix. Accordingly, c-Abl increases F-actin microspikes, but reduces cell spreading, thus revealing a sensory function during cell adhesion (Woodring et al., 2002). Plattner et al. (2003) suggested a positive role for c-Abl in the chemotactic response induced by PDGF. Accordingly, c-Abl has been firmly involved in dorsal ruffles induction, a process linked to cell invasion (Suetsugu et al., 2003). Indeed, cell invasion induced by PDGF is reduced in c-abl-deficient fibroblasts and restored by re-introduction of endogenous levels of cytoplasmic c-Abl.

Cytoplasmic Abl kinases have been implicated in human cancer. They are frequently de-regulated in human leukemia, in which they drive neoplastic transformation and cancer progression (Krause and Van Etten, 2005).

Interestingly, recent observations indicate that c-Abl and Arg are also de-regulated in solid tumors. For example, high cytoplasmic kinase activities have been detected in breast carcinomas (Srinivasan and Plattner, 2006) and non-small-cell lung cancers (Rikova et al., 2007). An increase in protein levels has been reported in breast carcinomas (Srinivasan and Plattner, 2006) and anaplastic thyroid cancers (Podtcheko et al., 2003); furthermore, Arg over-expression has been correlated with colon carcinoma progression (Chen et al., 1999).

c-Abl tyrosine kinase activity is tightly regulated in vivo. c-Abl kinase activity can be triggered by stimuli such as DNA damaging agents and integrin-mediated cell adhesion. The activation of c-Abl by ionizing irradiation is mediated by ataxia–telangiectasia mutated (ATM), presumably by direct phosphorylation of Ser-465 in the c-Abl kinase domain by ATM (Shafman et al., 1997). An important effect of c-Abl activation is cell cycle arrest: overexpression of c-Abl blocks cell cycle G1/S transition, and cells with compromised c-Abl function have deregulated cell cycle (Daniel et al., 1995). DNA damage also induces G1 cell cycle arrest, but whether c-Abl is required for DNA damage-induced G1 arrest is not clear.

Another effect of c-Abl activation is induction of apoptosis. Overexpression of c-Abl induces apoptosis, and c-Abl is apparently required for DNA damage-induced apoptosis: MCF-7 cells harboring
dominant-negative c-Abl and Abl/-/- fibroblasts are relatively more resistant to DNA damage-induced apoptosis (Yuan et al., 1997).

Mutant mice null for c-Abl and mutant mice with the C terminus of c-Abl deleted exhibit the same pleiotropic defects, including high neonatal death rate, defects in T cell and B cell development, reduced fertility, and developmental abnormalities in the spleen and bone.

Conclusions

From our obtained data put together, we can conclude that, considering gastric cancer and the c-abl gene expression, c-abl is down regulated inside tumor cells comparing to the normal gastric tissue from de resection limit (with no microscopic tumor invasion). This underlines the role of c-abl in normal tissue growth and its ability of inducing apoptosis when alteration of DNA occurs, as a result of different agents as stress, ionizing radiations. Down-regulation or loss of expression of c-abl is a fundamental event that leads at tumor apparition and progression.

The study of c-Abl in solid tumors has recently begun and there are few references regarding the relationship between c-Abl and gastric cancer. We are working to extend our group of patients to give more relevance to obtained data. Further study is required.

References


