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c-abl and YWHAZ gene expression in gastric cancer

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Abstract

This study aims to determine the gene expression for c-abl and YWHAZ in gastric cancer and the differences between the c-abl and YWHAZ gene expression inside the tumor *versus* healthy tissue (at the resection edges). This prospective study included 34 patients with gastric neoplasia, 21 men and 13 women, aged between 49 and 79 years (65.5 years median). After the surgical procedure, in these cases, we collected two tissue samples: one sample was obtained from inside the tumoral tissue and another sample from the gastric tissue, which was identified as normal apparently, as far as possible from the tumor (resection edge). For determining the c-abl and YWHAZ gene expression, we used the quantitative real-time polymerase chain reaction. Regarding the c-abl gene expression in gastric cancer, c-abl expression was identified as lower inside tumor cells comparing to the normal gastric tissue (resection limit). This difference of gene expression emphasize the role of the c-abl gene in normal tissue growth and the involvement in apoptosis induction when alteration of DNA occurs, as a result to different agents actions as stress, ionizing radiations. The loss of expression or even the down-regulation of the c-abl is a fundamental event that leads to genesis and progression of tumors. No significant differences of the YWHAZ gene expression between the tumoral and normal gastric tissue probes were recorded in our study.

Keywords: c-abl, YWHAZ, gastric cancer, resection edge.

Introduction

c-Abl is an enzyme that is involved in many cell processes, such as cell division. The gene for c-Abl is located 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 protein tyrosine kinase widely expressed inside tissues. It is activated by DNA damage. C-Abl is involved in apoptosis and the arrest of cell cycle as a response to DNA damage. However, the mechanisms of inducing these modifications are still unwell known.

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 tumor 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. Some reviews have discussed the mechanisms of cellular transformation by oncogenic and normal Abl kinases [1].

The role that c-Abl plays in human gastric carcinogenesis has been demonstrated by Cui *et al.*, studying Abelson interactor protein-1 (AB1), demonstrating that down-regulation of AB1 expression in human gastric carcinoma may play a critical role in tumor progression and patient's prognosis [2].

YWHAZ or 14-3-3 zeta is a gene located on the long arm of chromosome 8 and codifies a protein from 14-3-3 family, which mediates the signal transduced by binding proteins that contain phosphoserine. This family of proteins is highly conserved by plants and animals and is 99% identical in mouse, rat and sheep. YWHAZ is involved in cell cycle progression, recognition of DNA alterations, apoptosis, dynamic changes of cytoskeleton, control of gene expression transcription. There are few studies regarding YWHAZ gene expression in gastric cancer. Some authors [3, 4] have demonstrated in their studies that YWHAZ is up-regulated in gastric tumoral tissue compared to resection edge. In this study, we tried to evaluate YWHAZ pattern in Romanian gastric cancer patients.

Materials and Methods

Sample prelevation

The specimens used for this study were harvested from 34 consecutive gastric cancer patients, submitted to a gastric resection procedure (with curative or palliative intention) in the 2nd General Surgery Clinic, Emergency County Hospital, Craiova, Romania. The patient's age varied between 49 and 79 years, with a median of 65.5 years, with a gender distribution of 21 men and 13 women.

All collected tissues were pathologically assessed, on usual Hematoxylin–Eosin staining technique; the pathology report included the tumor's topography on the stomach, the histological type, the degree of differentiation, the tumor stage (T and N stage) and the resection margin status (invaded or not). After the surgical procedure was employed two samples of fresh tissue were collected in every case: one sample from the tumoral process (encoded with "A") and another sample as far as possible from the tumor, from the apparently normal gastric tissue (encoded with "B").

The probes were collected inside a tube containing RNA stabilization solution (RNAlater® Solution, Ambion), stocked at 4^0C for 12–24 hours and finally transferred at -80^0C.

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, USA). This technique uses the guanidine thiocyanate (GTC) and the β-mercaptoethanol, which inactivate the ribonucleases present in cellular extracts. GTC combined with SDS (sodium dodecyl sulfate) acts to destroy the nucleoproteic complexes, allowing the liberation of RNA in solution and its isolation, purified from proteins.

Dilution of the 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 the 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 (QRT-PCR)

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.

Reverse transcription of total RNA to complementary DNA

For reverse transcription of total monocatenar RNA to complementary DNA (cDNA), we have used the kit High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). 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. 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 (Table 1).

Table 1 – ***Protocol parameters of reverse transcription (temperature and time)***

Temperature [^{0}C]	25	37	85	4
Time [min.]	10	120	5	∞

Real-time polymerase chain reaction (RT-PCR)

At this stage, PCR products are synthesized from cDNA using TaqMan Gene Expression Master Mix and TaqMan probes Gene Expression assays (Applied Biosystems, Foster City, CA) specific for ABL1. PCR reaction was carried out in volumes of 20 µL. cDNA samples were diluted in water without nucleases (nucleated Free Water) at a concentration of 10 ng/µL cDNA. Final reaction mixture contained 80 ng cDNA (8 µL cDNA diluted). GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as endogenous control of the reaction. Each sample was analyzed in triplicate. Cycling was performed by Real-Time System Corbet 6200 HRM RotorGene (Table 2).

Table 2 – ***Polymerase chain reaction in real time – stages***

Stage	**UGD incubation**	**Activation AmpliTaq Gold, UP**	**PCR**	
			Cycle (50 cycles)	
	Storage	***Storage***	*Denaturation*	*Attaching/ Extension*
Time	2 min.	10 min.	15 s	1 min.
Temperature [^{0}C]	50	95	95	60

Results

We aimed to determine the differences between the expression of c-abl and YWHAZ inside the tumor and at the resection margins, in patients with gastric cancer. The relevance of the results has the histological diagnosis as a baseline, so we first defined the histological type of the tumor: out of the 34 patients with gastric neoplasms, 32 were diagnosed with gastric adenocarcinoma, two presented gastric adenocarcinoma mixed with neuroendocrine tumors and two patients presented gastric lymphoma with large cell. Two patients (Cases No. 6 and 8) with gastric adenocarcinoma have had microscopic tumor invasion at resection limits, therefore the results were considered irrelevant for these samples (no normal tissue to compare to).

Log2 was calculated and compared between the value obtained from the tumor and from the resection limit, respectively. The data obtained is presented in Figures 1 and 2, while the significance of the differences is presented in the Tables 3 and 4.

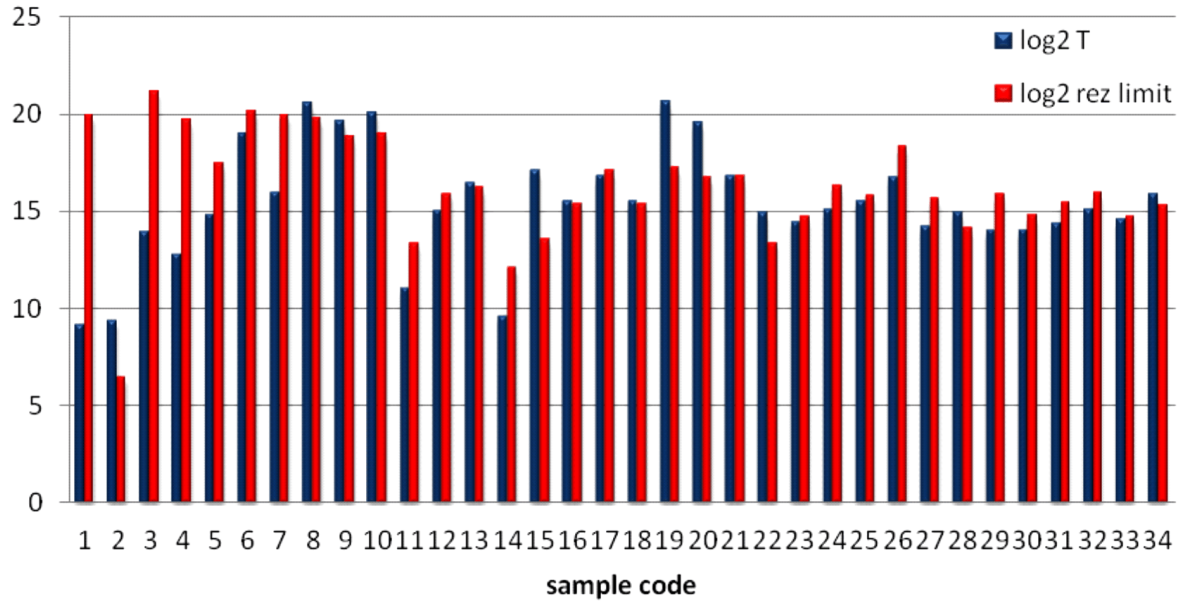

Figure 1 – *c-abl gene expression inside tumor compared to limit of resection (log2) (blue columns – log2 from tumor value and red columns – log2 from the resection limit).*

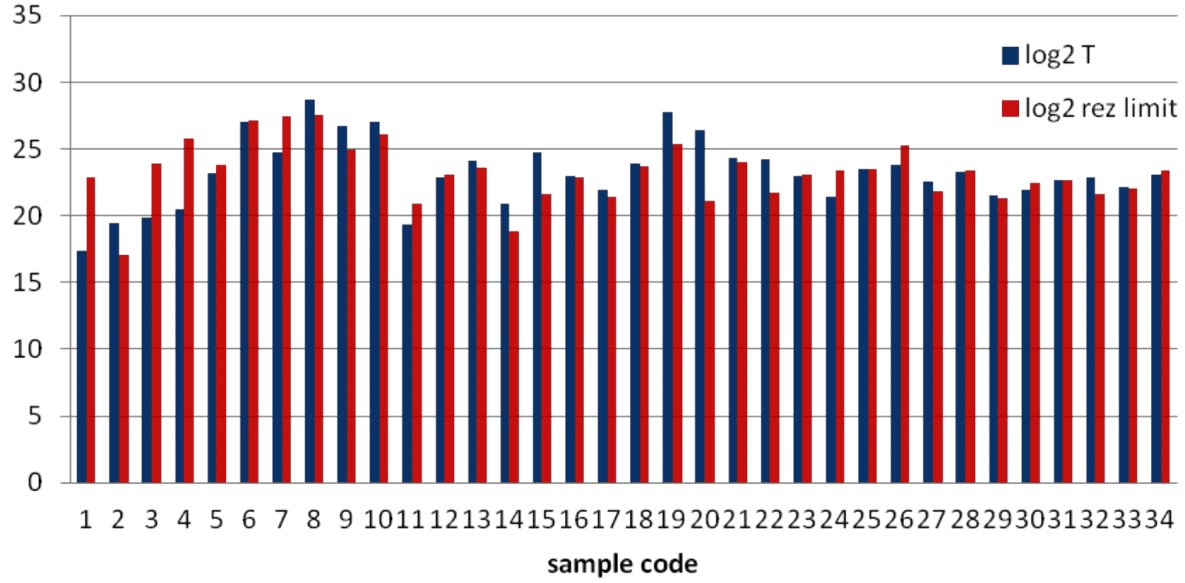

Figure 2 – *YWHAZ gene expression inside tumor compared to limit of resection (log2) (blue columns – log2 from tumor value and red columns – log2 from the resection limit).*

Table 3 – *Ratio value for c-abl gene expression in tumoral versus normal gastric tissue (significant gene expression differences are registered when ratio value is below 0.5 or higher then 2; no significant differences when ratio is between 0.5 and 2)*

Ratio value for c-abl gene	<0.5 (significant)	0.5–2 (non-significant)	>2 (significant)
All cases	14	15	5
Adenocarcinoma	14	12	3

Table 4 – *Ratio value for YWHAZ gene expression in tumoral versus normal gastric tissue (significant gene expression differences is registered when ratio value is below 0.5 or higher then 2; no significant differences when ratio is between 0.5 and 2)*

Ratio value for YWHAZ gene	<0.5 (significant)	0.5–2 (non-significant)	>2 (significant)
All cases	10	18	6
Adenocarcinoma	5	18	6

Two cases were gastric lymphomas (Nos. 2 and 8); the c-abl and YWHAZ gene expression presented a higher level inside the tumor tissue.

Two cases expressed mixed tumor cells inside – adenocarcinoma and neuroendocrine tumor (Nos. 15 and 20). In these cases, the c-abl and YWHAZ were overexpressed inside tumoral cells compared to the resection limit.

Out of the 34 cases included in the study, 29 were adenocarcinomas without tumoral invasion at the resection edge. Fourteen adenocarcinoma probes expressed a significant lower level of c-abl gene expression inside the tumoral tissue, 12 cases registered no significant difference of gene expression and in three cases c-abl was significantly higher expressed in the resection edge.

Out of 32 cases of adenocarcinomas, 20 cases had higher levels of YWHAZ gene expression inside tumoral tissue compared to resection edge, but the differences were significant only in five cases. Other six cases presented

a significant higher gene expression inside the resection edge. In 18 cases, there was no significant difference between gene expression in the tumoral tissue and at the resection edge.

We have calculated threshold cycle (Ct) that represents the point where the reaction reaches a fluorescence level higher than the background and becomes detectable. Threshold level is set in the exponential phase of amplification; the cycle when the reaction overpass threshold level is called threshold cycle. The obtained values are presented in Figures 3 and 4.

In the Figure 3, a higher level for threshold cycle at the tumoral level is noticed; this fact sustains and verifies a lower c-abl gene expression inside the tumor when compared to the resection edge. In 14 probes, the gene expression differences were not significant, but Threshold cycle value, in nine of these probes, was higher, sustaining the hypothesis that c-abl has lower expression in gastric tumoral tissue compared to the healthy tissue.

The Threshold cycle calculation for YWHAZ gene expression (Figure 4) reveals no significant differences between tumoral tissue and resection edge in most of the cases, thus we have concluded that there is no relation between YWHAZ gene expression and gastric carcinoma.

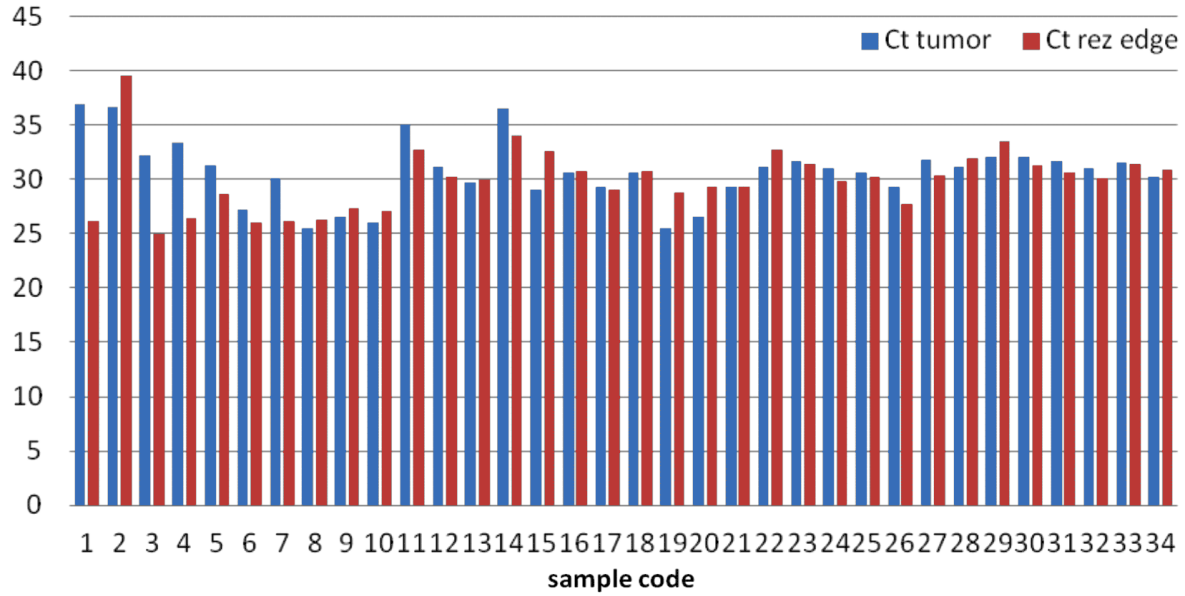

Figure 3 – *c-abl threshold cycle inside tumor compared to limit of resection (blue columns – tumor values and red columns – resection limit values).*

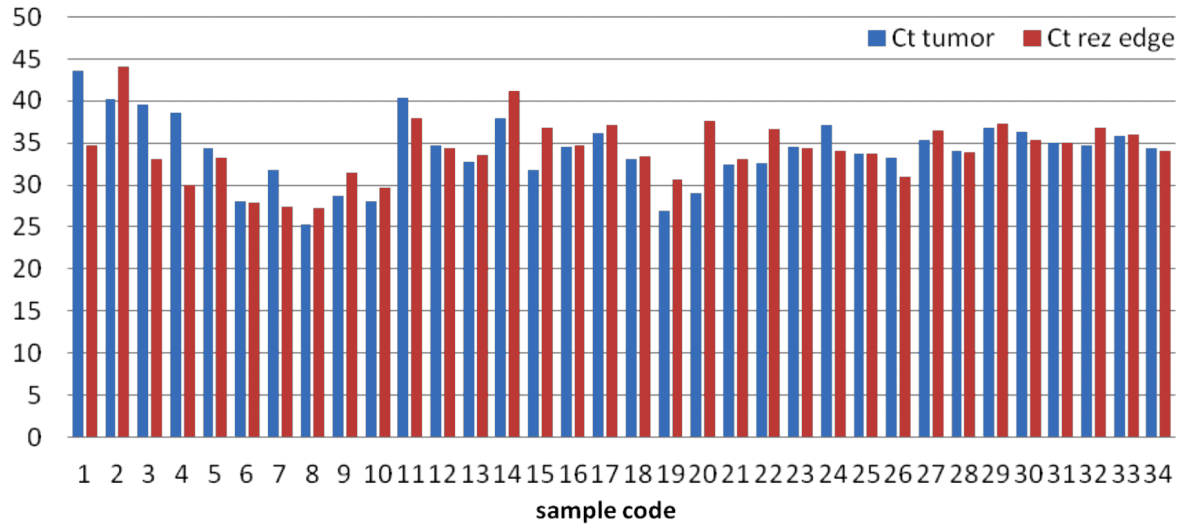

Figure 4 – *YWHAZ threshold cycle inside tumor compared to limit of resection (blue columns – tumor values and red columns – resection limit values).*

Discussion

The abl family of proteins was demonstrated to have a very important homeostatic role. The deletion of the abl gene and the absence of abl proteins can lead to multiple disorders that can determine the death of newborn animals, or severe diseases of lymphatic and bone systems.

The presence of c-Abl has been observed both at nuclear and cytoplasmic level. Nuclear c-Abl have been widely studied and its role well documented [5]. It is involved in regulating the cellular response when DNA damage occurs, as well as in inducing apoptosis and inhibition of cell growth. When DNA damage occurs, nuclear c-Abl is activated by the DNA-dependent protein kinase [6, 7]. On the other hand, the role of cytoplasmic c-Abl is not well known. There are some studies based on

biochemical and genetic findings that suggest a main role for cytoplasmic c-Abl and its involvement in morphogenesis and the dynamics of F-actin [8].

The role of c-Abl in F-actin dynamics was demonstrated, but its role in cell motility is not complete understood. c-Abl inhibits both cell adhesion and cell migration. Some studies have shown F-actin microspikes augmentation, condition that reduces cell migration. This suggests an important function for c-Abl in cellular adhesion processes [9].

Plattner *et al.* [10] suggested a positive role for c-Abl in the chemotactic response induced by growth factors. Some authors demonstrated the c-Abl involvement in cell invasion processes activated by dorsal ruffles induction [11]. Another characteristic of c-Abl is the suppressive effect on cellular proliferation. It might be possible that the negative effect on cellular proliferation to be determined by a cytotoxic effect [12, 13].

c-Abl with nuclear localization is involved in apoptosis being dependent of mechanisms triggered by tumor suppressor genes such as p53 and its homolog p73, as a response to DNA damage [14–16]. Other studies have demonstrated that the cytoplasmic form of c-Abl is activated in the cellular response to oxidative stress [17]. "Reactive oxygen species induce cytoplasmic c-Abl activity by a mechanism dependent on protein kinase Cδ (PKCδ)" [18].

Also, c-Abl seems to have different functions, depending on its localization. Subcellular nuclear c-Abl can determine the arrest of the cell cycle in G1-phase; by contrary, the cytoplasmic fraction of c-Abl can promote mitogenesis [19]. The cytoplasmic c-Abl has mitogenic role. It was found in cells with loss of Abl expression. Regarding these cells, it was observed a delay in DNA synthesis of 4–6 hours following growth factors induction [20, 21]. "c-Abl has been then identified as an important effector of Src for mitogenic signaling" [21], which is required for DNA synthesis.

The involvement of cytoplasmic Abl kinases in human cancers has been demonstrated. There is an association between these kinases de-regulation and neoplastic transformation and cancer progression in human leukemia [22].

Some studies regarding solid tumors have shown a de-regulation of c-Abl and Arg in these tumors. For example, high activity of cytoplasmic kinases has been identified in breast carcinomas [23] and non-small-cell lung cancers [24]. Increased protein levels have been reported in breast carcinomas [23] and anaplastic thyroid cancers [25]; furthermore, high expression of Arg has been correlated with progression of colonic carcinoma [26].

The *in vivo* activity of c-Abl tyrosine kinase is very well regulated. The agents involved in DNA damaging and cell adhesion mediated by integrins can become triggers for the activity of c-Abl kinase. Ionizing irradiation can also activate c-Abl mediated by ataxia–telangiectasia mutated (ATM); with high probability by direct phosphorylation of Ser-465 in the c-Abl kinase domain by ATM [27]. In this case, cell cycle arrest is the most important effect of c-Abl activation; the effect of this activation is blocking the G1/S transition of cell cycle due to the overexpression of c-Abl; this is the motive that when one encounter deregulated cell cycle expects to find cells with compromised c-Abl function [28]. One condition still remains unclear; the arrest of cellular cycle in G1 phase has been observed as a response of DNA damage, but is not sure if this process is strictly dependent by c-Abl activation.

Induction of apoptosis is another main effect of c-Abl activation. Induction of apoptosis has been observed in cells with overexpression of c-Abl, and c-Abl seems to be 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" [29].

Furlan *et al.*, have elucidated a molecular mechanism by which c-Abl participates in the oncogenic program driven by receptor tyrosine kinases; this might consist in identifying a new signaling signature to can permit the patients selection whose treatment may benefit from drugs that inhibit c-Abl expression [30].

de Oliveira *et al.* have demonstrated that, in addition to the well-known c-Abl kinase activity regulatory function, the "N-Cap-myristoyl tether may direct the c-Abl protein to anchor in the membrane as an additional mechanism to stabilize this N-terminal segment", condition that may occur in early signaling of induced apoptosis [31].

It is to be mentioned that there are few studies that investigate the relation between YWHAZ gene expression and gastric carcinoma. Most of them gather a small number of cases. At a lower level, ignoring the histological type of tumor, our study observes the overexpression of YWHAZ gene inside tumoral tissue in 20 of 34 cases with significant difference in 10 cases.

Data regarding a pattern of gene expression for YWHAZ are difficult to be evaluated, considering only the obtained results. After applying the exclusion criteria, the number of cases with significant differences of gene expression is smaller than the number of cases with no significant differences (11 *versus* 18).

There has been demonstrated the interaction between 14-3-3 proteins and a large number of oncogene products, tumor suppressor proteins, cell survival regulators, proliferation and growth, including the receptor of the growth factor 1 insulin-like, p53, PI3K and other components of signaling pathway mediated by growth factors [32, 33].

Jang *et al.*, using proteomic analyze, has identified high levels for various proteins among which 14-3-3, isoform zeta, in 18 patients with gastric adenocarcinoma using two tissue probes from same patient, one from tumoral tissue, the other from the resection edge, considered normal tissue [34]. There are few data regarding YWHAZ high gene expression inside oral cancers and pulmonary adenocarcinoma [35–37].

Recent studies emphasize the YWHAZ role in decreasing cell adhesion in mammary tumors by TGF-β/Smads way of signaling activation. High expression of ErbB2 and YWHAZ determine augmentation of cell migration and decreasing of cellular adhesion, two conditions that are required for tumor cells to invade nearby tissues [38].

It is possible that YWHAZ to serve as biomarker of inflammation associated to neoplasia, without taking into account his functions, while activated monocytes and

macrophages are at high level at peritoneal level of patients with advanced epithelial ovarian carcinoma [39]. It is not yet proven the paracrine role of 14-3-3 zeta in ovarian epithelial carcinoma. Similar, we cannot demonstrate a correlation between YWHAZ gene expression and gastric cancer.

In a study, Fang *et al.* have found an association between overexpression of YWHAZ and doxorubicin-resistant mammary cancerous cells [40].

A recent study on gastric cancer [3] reveals that overexpression of the YWHAZ protein was frequently detected in gastric cancer cell lines. This overexpression significantly correlates with greater tumor volume, venous and lymphatic invasion, advanced stage of tumor, and higher recurrence rate. Patients presenting YWHAZ-overexpression inside their tumors have lower long time survival rates. In multivariate analysis, YWHAZ was associated with a worse outcome in patients with various tumors.

Lin *et al.* have reported a high global gene expression for YWHAZ at DNA, mRNA and protein level in patients with head and neck squamous carcinoma. They demonstrate YWHAZ ability to control cellular growth when is experimentally up-regulated or down-regulated being a cell cycle regulator [41].

YWHAZ involvement in gastric cancer has been poorly understood according to our data. The gene expression inside tumoral tissue compared to resection edge represents the start point for correlating this gene with gastric cancer. Although there are many studies that showed the YWHAZ gene expression correlation with gastric carcinoma, we demonstrated through our results that there seems not to be significant differences of gene expression between the two studied probes; most of the cases have shown small difference of value inside tumor compared to the resection edge.

Conclusions

From our data, 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 (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, because of different agents as stress, ionizing radiations. Down-regulation or loss of expression of c-abl is a fundamental event that leads to tumor genesis and progression. The YWHAZ gene expression and gastric cancer seem not to be correlated; our data have showed no relevant differences of gene expression between tumoral and healthy gastric tissue. We cannot conclude that there is a certain pattern of YWHAZ gene expression regarding gastric adenocarcinoma, but we have registered a significantly higher level of gene expression inside tumor tissue in other cases but adenocarcinoma: two gastric lymphomas with large cells and two gastric adenocarcinomas mixed with neuroendocrine tumors.

Conflict of interests

The authors declare that they have no conflict of interests.

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