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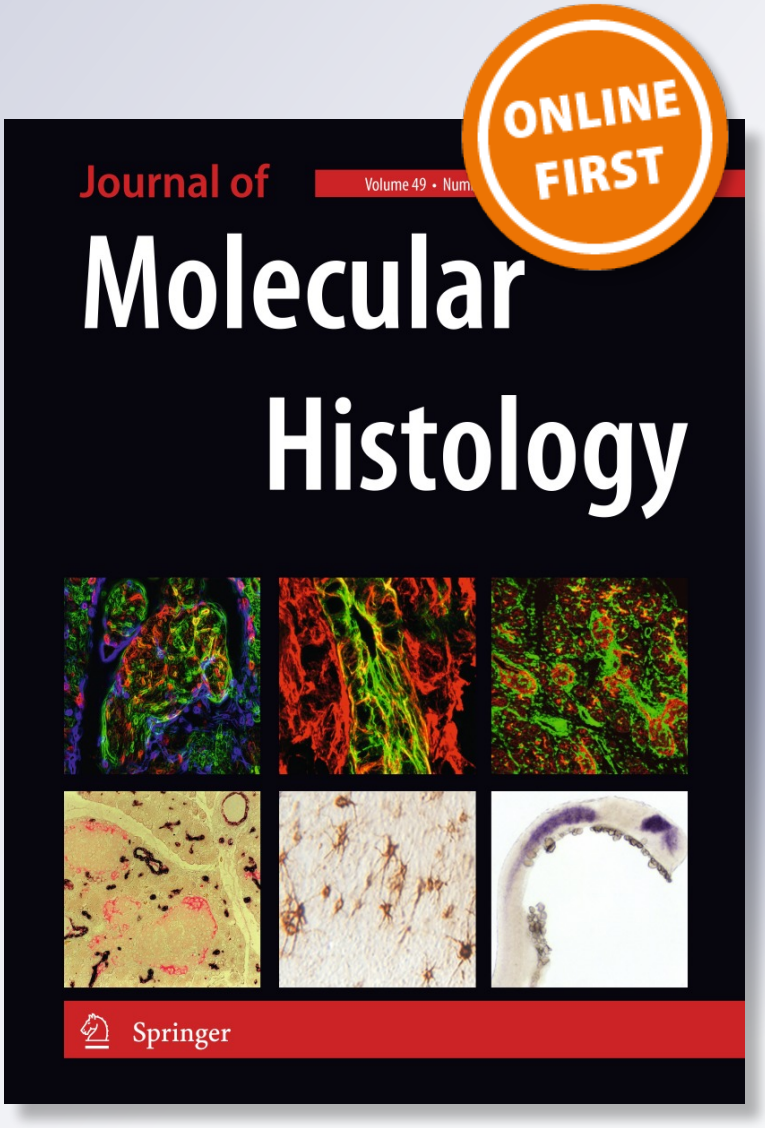

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SHORT COMMUNICATION

VEGFA and VEGFR2 RNAscope determination in gastric cancer

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Abstract
Gastric cancer is the fifth most common cancer and third leading cause of cancer-related death worldwide. Several studies on angiogenic blocking agents in gastric cancer revealing promising results by the use of monoclonal antibodies against VEGFA or its receptor VEGFR2 or against VEGFA activating pathway. The validation of biomarkers useful to better organize the clinical trials involving anti-angiogenic therapies is crucial. Molecular markers such as RNA are increasingly used for cancer diagnosis, prognosis, and therapy guidance as in the case of the targeted therapies concerning the inhibition of angiogenesis. The aim of this study is to set the conditions for evaluating the expression of VEGFA and VEGFR2 in gastric cancer specimens and in healthy gastric mucosa by the use of RNAscope, a novel RNA in situ hybridization (ISH) method that allows the visualization of a specific gene expression in individual cells. We found the increased expression of VEGFA in the tubular glands and VEGFR2 in the endothelium of gastric cancer samples mainly in the T2, T3 and T4 stages of tumor progression as compared to the healthy controls. These results obtained by the application of this highly sensitive method for oligonucleotide detection the role of angiogenesis in gastric cancer progression already highlighted by conventional immunohistochemical methods, and offer significant promise as a new platform for developing and implementing RNA-based molecular diagnostics also in the conditions in which immunohistochemistry is not applicable.

Keywords Gastric cancer · Immunohistochemistry · RNAscope · VEGFA · VEGFR2

Introduction

Gastric cancer is the fifth most common cancer and third leading cause of cancer-related death worldwide according to the data from Global Cancer Statistics, 2012 (Torre et al. 2015). Surgery and perioperative treatments are potentially curative for patients with resectable cancer but nowadays, the prognosis for advanced gastric cancer is poor and although chemotherapy remains the leading treatment for patients with advanced disease, targeted therapy might be an option to improve the prognosis (Schulte et al. 2014).

One of the targeted therapies concerns the inhibition of angiogenesis according to different literature evidences demonstrating a close relationship between angiogenesis and cancer (Maeda et al. 1995b). It has been hypothesized that cancer cells begin to promote angiogenesis early in tumorigenesis and the angiogenesis stimulates tumor growth and progression (Folkman 1990). The angiogenic switch is characterized by oncogene-driven tumor expression of pro-angiogenic proteins, among them vascular endothelial growth factor A (VEGFA) has been identified as the most potent cytokine involved in tumor angiogenesis and metastasis formation. The activity of VEGFA is mediated by two tyrosine kinase receptors, VEGFR-1 and VEGFR-2 which differ considerably in signalling properties. Some effects of VEGFA include the enhancing of the vascular permeability (Dvorak et al. 1995), the stimulation of serine protease or metalloproteases production (Pepper et al. 1991; Unemori et al. 1992), and the inhibition of apoptosis of endothelial cells (Gerber et al. 1998).

VEGFA expression has been demonstrated increased in many solid tumors including gastric cancer. Maeda et al. studied the correlation between VEGFA expression, microvascular

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density and clinicopathologic factors in gastric carcinoma specimens (Hanahan and Folkman 1996; Tanigawa et al. 1997). They found a positive correlation between VEGFA expression, hepatic metastasis and poor prognosis. Moreover, the same authors analysed the relation between p53 and VEGFA in gastric cancer and concluded that the 5-year survival rate was lowest in the patients with p53 and VEGFA positive tumors with respect to none or only VEGFA positivity (Maeda et al. 1998). Takiushi et al. observed that immunohistochemical VEGFA evaluation constituted a useful predictor of chemotherapy response in unresectable gastric carcinoma (Takiuchi et al. 2000). Moreover, a recent meta-analysis study revealed that VEGFR2 overexpression is a promising negative prognosis predictor for patients with gastric cancer (Li et al. 2018). Due to VEGFA/VEGFR2 importance in tumor angiogenesis several inhibitors are undergoing clinical testing in several malignancies, including Bevacizumab, a monoclonal antibody anti-VEGFA (Kim et al. 2009); Ramucirumab, a fully humanized IgG1 monoclonal antibody specifically blocking the extracellular domain of VEGFR2 (Lu et al. 2003); Sunitinib, an oral multitargeted tyrosine kinase inhibitor of VEGFRs (Bang et al. 2011); Apatinib is a tyrosine kinase inhibitor selectively targeting VEGFR2 (Scott 2018).

In this study, we have therefore used for the first time an mRNA in situ hybridization technique called RNAscope to detect VEGFA and VEGFR2 mRNA expression levels in the tumor cells lining the tubular glands and in the microvascular endothelium of gastric cancer specimens at different stages of invasiveness and in healthy controls. It is well known the expression of VEGFA by stromal and inflammatory cells and its role during the tumor progression (Longo et al. 2018; Ribatti et al. 1999; Roskoski 2007), so we focused on the tubular gland component of gastric tissue, excluding the stromal cells.

Although in situ RNA detection technique such as the classical ISH has been used in the past to study VEGF expression in metastatic gastric cancer (Takahashi et al. 2002) this approach lack robustness and sensitivity to reliably detect the expression of most human genes. RNAscope technology is a revolutionized and novel ISH derived assay for detection of target RNA within intact cells. This assay is known for its sensitivity and specificity due to unique probe design strategy that allows simultaneous signal amplification and background suppression to achieve single-molecule visualization while preserving tissue morphology. Moreover its versatility allows it to be used by both fluorescence and visible approach (Wang et al. 2012).

Materials and methods

Patients and data collection

Tissue samples from 30 patients with gastric carcinoma who underwent curative resection of primary tumor and their paired adjacent normal gastric mucosa were collected from the archive of the Section of Pathology of the University of Bari, Hospital Policlinico, Bari, Italy. Full ethical approval and signed informed consent from individual patients were obtained to conduct the study. All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and later versions. Tumors were divided into three histological subgroups, T1, T2 and T3/T4, based on T level score indicating the cancer invasion into through the 5 layers of the stomach wall according to TNM system. 10 control specimens were taken from non-tumour gastric mucosa without hyperplasia or atypical hyperplasia at 5 cm away from the edge of a tumour specimen. Tissue samples were fixed in formalin and embedded in paraffin according to standard procedures. 4-µm-thick sections were cut and mounted on glass slides.

VEGFA, VEGFR2 RNAscope assay

RNAscope assay was performed on FFPE biopsies using RNAscope 2.5 HD Reagent Kit [RED 322360, Advanced Cell Diagnostics (ACD), Hayward, CA]. Briefly, tissue sections were deparaffinized with xylene and 100% ethanol and incubated with pretreat-1 solution for 10 min, pretreat-2 for 15 min, and pretreat-3 for 30 min (Pretreatment kit 322330, ACD). The slides were then hybridized with a probe Hs-VEGFA (ref. 310061), Hs-VEGFR2 (ref. 312121), positive control probe - Hs-PPIB (ref. 313901), negative control probe - DapB (ref. 310043) in the HybEZ oven (ACD) at 40 °C for 2 h. The Hs-PPIB probe for human housekeeping gene PPIB was used as a control to ensure RNA quality. After hybridizations, slides were subjected to signal amplification using HD 2.5 detection Kit, and hybridization signal was detected using a mixture of Fast- RED solutions A and B (1:60). After counterstaining with Gill's hematoxylin, slides were dried in a 60 °C dry oven for 15 min and mounted with Ecomount (BioCare Medical, EM897L). Sections from each experimental group were scanned using the whole-slide morphometric analysis scanning platform Aperio Scanscope CS (Leica Biosystems, Nussloch, Germany). All the slides were scanned at the maximum available magnification (40×) and stored as digital high resolution images on the

workstation associated with the instrument. Based on the PPIB evaluation, all the cases were included in the analysis. Digital slides were inspected with Aperio ImageScope v.11 software (Leica Biosystems, Nussloch, Germany) at 20× magnification and ten fields with an equal area were selected for the analysis at 40× magnification. As concerns the VEGFA we have selected the tumor cells present in tubular glands. The mRNA expression was assessed with the Positive Pixel Count algorithm embedded in the Aperio ImageScope software and reported as positivity percentage, defined as the number of positively stained pixels (the Hscore is automatically calculated) on the total pixels in the image. This algorithm usually used to quantify the amount of a specific stain present in a scanned slide image in base to the color specified and three intensity ranges (weak, positive, and strong), it has been set in order to calculate the relative contribution of the chromogen at every pixel location providing precise information on the distribution of biomarkers in the tissue. Moreover, the pseudo-color markup generated image allowed us to tune the parameters until the algorithm results are sufficiently accurate in order to exclude the background staining from the analysis and the cluster derived signals thus to distinguish the specific stain. The statistical significance of differences between the mean values of the percent labeled areas between tumor specimens and control tissues was determined by the 2 way Anova test in GraphPad Prism 5.0 software (GraphPad software, La Jolla, CA, USA). Findings were considered significant at P values < 0.05.

Results

VEGFA expression

The RNAscope assay was performed to evaluate the expression of VEGFA mRNA in tumor cells lining tubular glands in T1, T2, T3/T4 gastric cancer stages and control samples (Fig. 1a–d). A low expression with sporadic dots is observed in control samples (Fig. 1a) and a little higher in T1 cancer samples (Fig. 1b). As concern the VEGFA expression in T2 and T3/T4 gastric cancer specimens (Fig. 1c, d) the amount of positive cells is much more high respect to the controls (Fig. 2a). The inset in the Fig. 1d shows a detail of a VEGFA positive tumor cell containing dots (arrow) and clusters (arrowhead) after RNAscope staining. Morphometric analysis allowed us to determine a significant little difference in VEGFA expression in T1 (1.5% ± SE 0.1%) and in T2 (6.2% ± SE 0.18%) and T3/T4 (7% ± SE 0.6%) gastric cancer samples compared with controls (0.6% ± SE 0.04%) (Fig. 3 a).

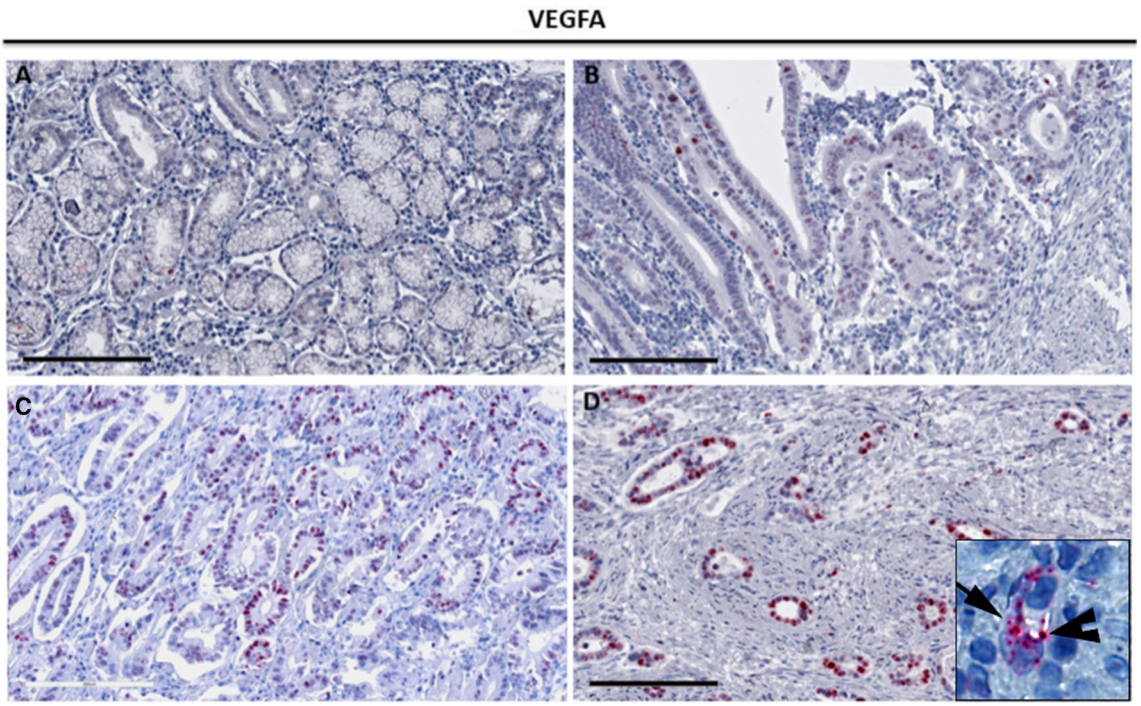

Fig. 1 VEGFA mRNA evaluation in tumor cells lining tubular glands in histological control samples CTRL (**a**), and gastric carcinoma T1 (**b**), T2 (**c**) and T3/T4 (**d**) tumor samples. RNAscope assay shows that VEGFA messenger expression in tumor samples (**b**–**d**) is increased as compared to CTRL (**a**). The inset shows a detail of a VEGFA positive tumor cell containing dots (arrow) and clusters (arrowhead) after RNAscope visible staining. Positive cells are indicated by arrows. Scale bar: **a**–**d** 200 µm

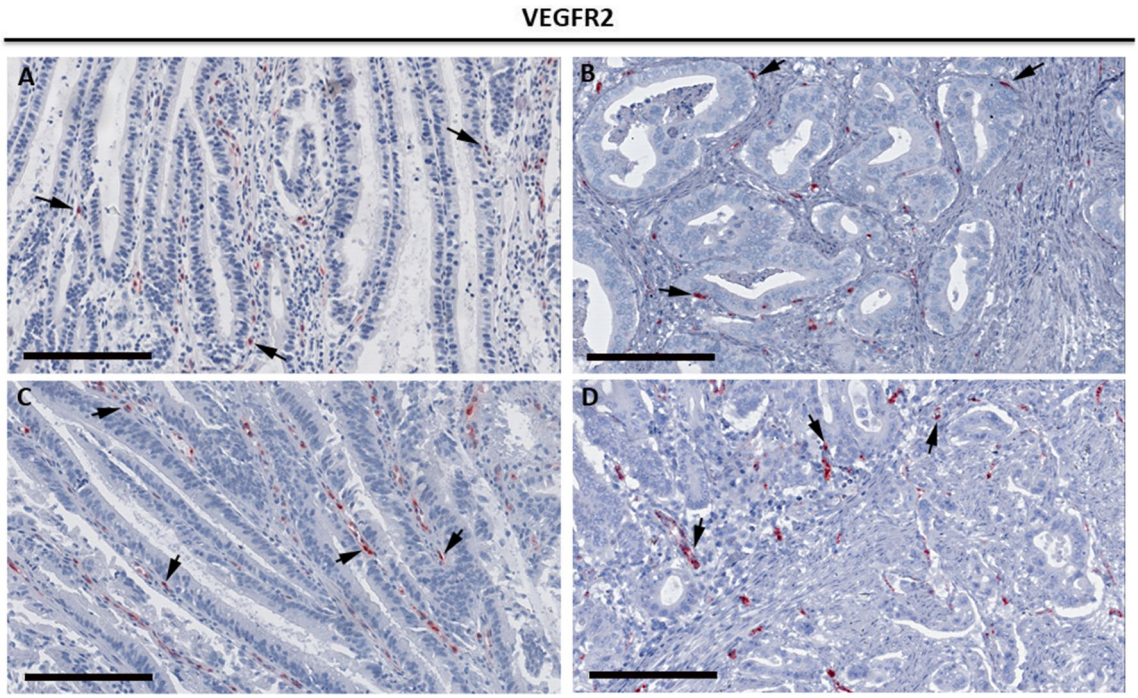

Fig. 2 VEGFR2 mRNA evaluation in histological control samples CTRL (**a**), and gastric carcinoma T1 (**b**), T2 (**c**) and T3/T4 (**d**) tumor samples. RNAscope assay shows that VEGFR2 messenger expression in the microvascular endothelium of the tumor samples (**b–d**) is increased as compared to CTRL (**a**). Positive cells are indicated by arrows. Scale bar: **a–d** 200 µm

VEGFR2 expression

To assess the VEGFR2 expression pattern in endothelial cells, RNAscope assay was performed in T1, T2, T3/T4 gastric cancer stages and control samples. A low expression signals derived from endothelium has been seen in control gastric sample (Fig. 2a), while a strong increase of VEGFR2 expression in T1, T2 and T3/T4 gastric cancer specimens (Fig. 2b–d) was detected. Morphometric analysis showed a significant difference in VEGFR2 expression in T1 (0.23% ± SE 0.05%), T2 (0.45% ± SE 0.06%), T3/T4 (0.74% ± SE 0.08%) gastric cancer compared with controls (0.03% ± SE 0.01%) (Fig. 3b).

Discussion

Cancer is the second leading cause of death worldwide. As regards the gastric cancer, a statistical study showed that in 2015, there were 1.3 million incident cases of stomach cancer and 819,000 deaths worldwide (Global Burden of Disease Cancer et al. 2017). Although diagnosis and perioperative therapy have improved over the last decades, outcome is still poor with overall 5-year survival rates of less than 40% (Sisic et al. 2018). Due to the fail in response to the classical chemotherapy for inoperable or advanced-stages, other treatments are being tested to improve the survival in gastric adenocarcinoma and the antiangiogenic therapy is one of the biologically targeted approaches that are giving promising results (Jin and Yoon 2017).

Tumor angiogenesis plays a crucial role in the growth, invasion, and metastatic spread of solid neoplasms by facilitating the delivery of oxygen, nutrients, and growth factors to tumor cells (Macedo et al. 2017; Nandikolla and Rajdev 2016). Although angiogenesis is highly complex process, VEGFA/VEGFR2 signaling mediates the main effects on angiogenesis (Ribatti et al. 2016; Senger et al. 1983). Several studies on angiogenic blocking agents in gastric cancer are emerging during last years revealing promising results by the use of monoclonal antibodies against VEGFA or its receptor VEGFR2 or against VEGFA activating pathway (Jia and Cai 2016).

Here, we have described the validation of RNAscope, a novel non radioisotopic RNA *in situ* hybridization technology in the study of VEGFA and VEGFR2 expression in human gastric cancer.

VEGFA is thought to contribute to tumor growth by promoting angiogenesis and stroma formation, both directly, through its action as an endothelial cell growth factor, and indirectly, by increasing vascular permeability. This evidence derives by the detection of VEGFA/VEGFR2 in gastric cancer indicating that primary autochthonous human tumors of gastrointestinal origin regularly express both VEGFA at molecular and protein levels and

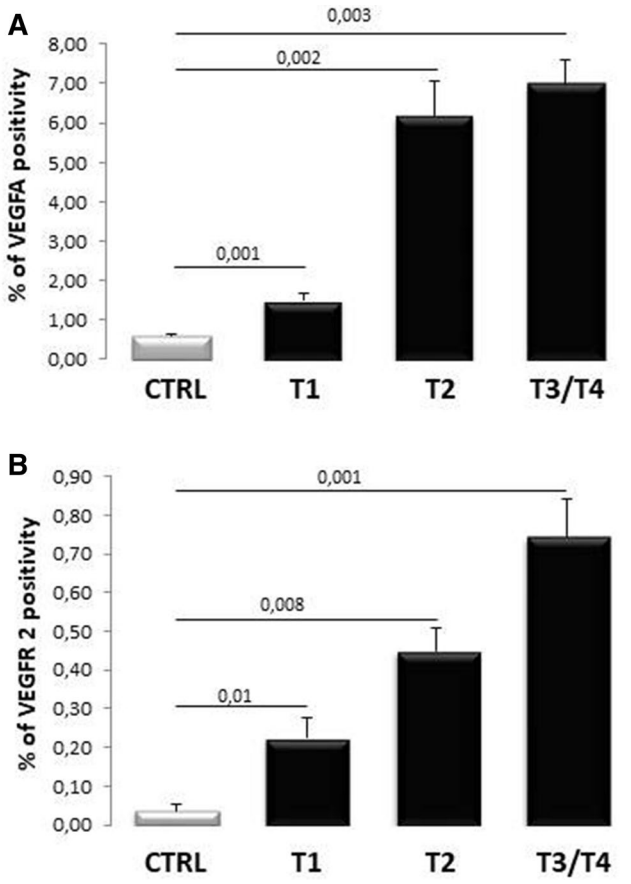

Fig. 3 Morphometric analysis of VEGFA (**a**) and VEGR2 (**b**) mRNA positivity in gastric carcinoma samples and in controls (CTRL). The per cent of VEGFA and VEGFR2 mRNA expression significantly increases in all the tumor samples compared to CTRL (**a**)

that adjacent stromal vessels express VEGFR2 (Brown et al. 1993). Microvessel density represents a good prognostic indicator and may be useful as a predictor for the mode of recurrence in patients with gastric carcinoma, through the direct correlation between angiogenesis, the tumor stage, and VEGFA expression (Maeda et al. 1995a, b, 1996). Other studies have confirmed these data (Araya et al. 1997; Nienhuser and Schmidt 2017; Tanigawa et al. 1997; Yamamoto et al. 1998). Moreover, VEGFA/VEGFR2 are present in the supernatant of various human gastric carcinoma cell lines, and the activation of VEGFA signaling facilitates gastric tumor growth and metastasis (Chen et al. 2016).

Molecular markers constituted by DNA, RNA and proteins are increasingly used for cancer diagnosis, prognosis, and therapy guidance (Hamburg and Collins 2010), and the validation of robust biomarkers able to screen the patients responsive to the treatments to better organize the clinical trials involving anti-angiogenic therapies is imperative (Caporarello et al. 2017).

Among the biomarkers classes, those based on RNA have emerged as the major class of cancer markers (van 't Veer et al. 2002). Also if real-time PCR is considered the gold standard platform in gene expression analysis the destruction of the tissue occurring during the sample processing prevent to localize the obtained signals to individual cells. RNAscope is a novel RNA ISH method that allows the visualization of a specific gene expression in individual cell through the use of a novel probe design strategy and a hybridization-based signal amplification system to simultaneously amplify signals and suppress background (Wang et al. 2012). In this study, we have used the RNAscope technique in order to visualize and measure the positivity in expression of VEGFA and VEGFR2, in gastric cancer specimens and in healthy gastric mucosa focusing our analysis on VEGFA-positive tumor cells lining the tubular glands and VEGFR2-positive endothelial cells. Our data showed the increased expression of VEGFA in the tubular cancer cells and the increased VEGFR2 expression in the endothelium in the intermediate and advanced progression stages of gastric carcinoma. These results corroborate the important role of the angiogenic response during the tumor progression found from other authors trough immunohistochemical analysis and its role as a predictor during tumor progression. Moreover, VEGFA and VEGFR2 seem to be good candidates as biomarkers to be used in the clinical trials involving anti-angiogenic therapies. Finally this study allowed us to set the experimental conditions to evaluate the expression of these markers by the RNAscope.

The data obtained let us to conclude that RNAscope represents a significant improvement in RNA ISH methodology and is compatible with clinical sample types and laboratory workflows. The utilization of this highly sensitive method for oligonucleotide detection offers significant promise as a new platform for developing and implementing RNA-based molecular diagnostics useful when IHC is not available or when there is a difficulty in standardizing immunohistochemical analysis as occurs during anti-VEGF therapy in invasive gastric cancer. The results of this study obtained by means of RNAscope are in agreement with other literature data concerning the correlation between angiogenesis and gastric cancer progression evaluated through other techniques including the classic IHC.

Moreover, the combination of mRNA analysis through in situ hybridization and protein analysis through immunohistochemistry in the same section could be an extremely powerful technique that allows to identify specific cell populations. when IHC is not available or when there is a difficulty in standardizing immunohistochemical analysis as during anti-VEGF therapy in invasive gastric cancer (Jia and Cai 2016), and our data constitute the basis that deserve to be deepened in future experiments. Finally, it should be taken in account that RNAscope has several disadvantages;

in fact, if the samples are of poor fixation quality, they will not be well stained and the cost is much higher compared to conventional immunohistochemistry.

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Compliance with ethical standards

Conflict of interest All authors have seen and approved the manuscript being submitted and have no conflict of interest to declare.

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