

## NF2 expression levels of gastrointestinal stromal tumors: a quantitative real-time PCR study

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### ABSTRACT

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Gastrointestinal stromal tumors are the most common mesenchymal tumors of the gastrointestinal tract. Until today, there have been few markers specific for the tumor. This has complicated the differential diagnosis of the neoplasm from tumors of smooth muscle origin. Recently, the proto-oncogene c-kit has been shown to be a very relevant marker as it almost invariably is expressed in gastrointestinal stromal tumors. Radiation exposure, hormonal and genetic factors, particularly neurofibromatosis 2, have been implicated in their development and growth. GIST initiation, either in NF2-associated or in sporadic cases, is linked to inactivation of members of the proteins 4.1 superfamily. The majority of the mutations identified in the NF2 gene result in a truncated protein and are clinically associated with a severe phenotype. Occasionally, missense mutations associated with a mild phenotype may occur. We compared NF2 gene expression in 5 cases with gastrointestinal stromal tumors by quantitative real-time polymerase chain reaction analysis. NF2 gene mRNA expression was assessed in fresh tissue of stomach from 5 consecutive patients. We detected no alterations in NF2 gene expression in the quantitative analyses of the 5 tumors.

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### Introduction

Gastrointestinal stromal tumors (GIST) are the most common mesenchymal tumors of the digestive tract. The constitutive activation of the c-kit proto-oncogene is a characteristic genetic alteration in GIST, but alterations in chromosomes 14 and 22 may also play a role in the molecular pathogenesis. Radiation exposure, hormonal and genetic factors, particularly neurofibromatosis 2 (NF2), have been implicated in their development and growth. GIST initiation, either in NF2-associated or in sporadic cases, is linked to inactivation of members of the protein 4.1 superfamily, the merlin/schwannomin, an NF2 gene product. Precisely, about 10% of sporadic GIST are caused by loss of heterozygosity (LOH) on chromosome 22q12 of the NF2 tumor suppressor gene, whereas no causative gene is known for the remaining 40%<sup>1-8</sup>.

Within the 4.1 superfamily, merlin shares the highest degree of homology with a subgroup of proteins including ezrin, radixin, and moesin (ERM proteins), linking the actin cytoskeleton (by an actin-binding region in the C-terminus) to the cell membrane glycoproteins such as the CD44 glycoprotein (by its N-terminal residues) in polarized cells. The N- and C- terminal halves of ERM proteins mutually interact intramolecularly to suppress their binding activities. The C-terminal threonine phosphorylation maintains ERM proteins in the active state by suppressing the intramolecular interaction<sup>5,7,9,10</sup>.

The region of merlin with the greatest structural similarity to the ERM proteins correspond to two-thirds of its N-terminal. The C-terminus of merlin is unique and lacks the conventional actin-binding region of the ERM proteins but it interacts with F-actin through the N-terminus<sup>5,9,11-14</sup>.

The similarity of merlin to the ERM proteins indicates that its function might be re-

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lated to those of the ERM proteins: organization of membrane extensions and cell adhesion, membrane traffic and cell signaling<sup>15</sup>. Indeed, merlin interacts, either directly or indirectly, with components of cadherin-mediated cell junctions and with a number of proteins that may influence cell growth regulation including paxillin, erbB2, p21-activate kinase, and p53<sup>5,7,9,10,13</sup> through MDM2 degradation<sup>16</sup>.

Several experimental studies have demonstrated that merlin overexpression results in a significant decrease in cell proliferation, reversion of Ras-induced transformation, and reduced tumor formation in nude mice. In contrast, inactivation of the NF2 gene leads to the development of cancer through loss of contact-dependent inhibition of growth<sup>13,14,17-20</sup>.

Most of the mutations identified in the NF2 gene result in a truncated protein and are clinically associated with a severe phenotype. Occasionally, missense mutations associated with a mild phenotype may occur.

In this report, we compare NF2 gene expression in 5 cases of GIST by quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) analysis. NF2 gene mRNA expression was assessed in fresh tissue of stomach from 5 consecutive patients. RNA amplification was assessed by RT-PCR and measured quantitatively. Our purpose was to further investigate the possible role of merlin in GIST tumorigenesis and progression.

## Methods and results

Tissue specimens were obtained from 5 consecutive patients affected by GIST surgically treated at the Department of Surgical Pathology (Careggi Hospital, Florence, Italy) during the period August 2005 to July 2006, in which fresh tumor tissue was available for RT-PCR analysis. Two samples were from men and 3 from women. The average age at the time of the surgery was 54 years.

From each fresh surgical specimen, we selected a fragment macroscopically representative of the lesion. Successively, we cut it in half: from 1 half several 5- $\mu$ m frozen sections stained with hematoxylin-eosin were obtained to verify the adequacy of the specimens selected for RT-PCR (presence of pathological tissue only); the other half was immersed in RNAlater<sup>TM</sup> (QIAGEN, Hilden, Germany), so kept overnight at +4 °C, and finally stored at -80 °C until analyzed.

The thawed specimens were cut in small pieces and homogenized. After proteinase K digestion (250  $\mu$ g/ml for 1 h at 37 °C), total RNA was isolated with 6100 Nucleic Acid PrepStation (manufacturer's protocol). Total RNA (500 ng) was subject to reverse transcription to cDNA using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) (manufacturer's protocol). We extracted RNA from all samples, and the RNA concentration and purity in preparations was quantitated

spectrophotometrically by measuring their absorbance at 260 nm and 280 nm. The RNA fragmentation state was estimated with ethidium bromide-stained 1% agarose gel.

Real-time quantitative PCR was performed on an ABI PRISM 7000 Sequence Detector System (Applied Biosystems). PCR products for NF2 were detected using gene-specific primers and probes labeled with reporter dye FAM (Assay on Demand, Applied Biosystems). GAPDH was used as endogenous control gene for normalization.

PCR reactions were carried out in 96-well plates with 20  $\mu$ l per well using 1x TaqMan Universal PCR Master-Mix. After an incubation for 2 min at 50 °C and 10 min at 95 °C, the reaction continued for 50 cycles at 95 °C for 15 sec and 60 °C for 1 min. The  $2^{-\Delta\Delta C_t}$  method described by Livak *et al.*<sup>21</sup> was used to calculate fold expression levels relative to the average value of all the RNA specimens. For each sample of GIST, a specimen of normal mucosa from the same patient was used as control sample.

NF2 expression in all GIST was compared to the average value of expression (reference value) of all the benign tissue. The mean of NF2 relative expression of all GIST showed an expression higher (4.9%) than the average of all tissue used as calibrator ( $P = 0.05$ ).

The shift of expression level of NF2 as estimated through relative RT-PCR was calculated according to the Wilcoxon Mann Data analysis was performed using the SPSS Version 15 (Chicago, IL) statistical package. A  $P$  value  $\leq 0.05$  was considered to be statistically significant.

After drawing of the small samples for RT-PCR, the remaining tissues were routinely fixed in 10% buffered formalin and embedded in paraffin. Five- $\mu$ m-thick sections were stained with hematoxylin-eosin for the morphological evaluation. A c-kit analysis was performed, and all cases resulted positive. Diagnostic criteria used were those indicated by the most recently revised World Health Organization (WHO) Classification of gastrointestinal stromal tumors.

## Discussion

GIST are the most common mesenchymal tumors of the gastrointestinal tract<sup>22</sup>. The few markers specific for GIST has complicated the differential diagnosis of the neoplasm from tumors of smooth muscle origin. The proto-oncogene c-kit has been recently shown to be a very relevant marker, as it is almost invariably expressed in GIST<sup>23-25</sup>. Mutations of the gene are frequently detected in human GIST, and transfection of the mutant c-kit into murine lymphoid cells has been shown to induce malignant transformation. This is evidence that the mutation of c-kit may contribute to the tumorigenesis of GIST<sup>24,26</sup>. However, as only about half of GIST show c-kit mutations<sup>27-29</sup>, alternative mechanisms leading to malignant transformation of GIST are possible.

Molecular studies have shown that several different genetic changes occur in GIST. The most frequent alterations in comparative genomic hybridization (CGH) and LOH studies have been revealed to be losses of chromosomes 22q and 14q, both in benign and malignant tumors<sup>30-32</sup>. In line with the CGH results indicating losses of 22q, both in tumors with high mitotic activity as well as in tumors with low mitotic activity, which is an indicator of potential malignancy of these tumors. Interestingly, LOH was significantly more frequently detected in tumors with high mitotic activity. Thus, allelic loss at these chromosome sites may be a common and primary abnormality responsible for the development of GIST.

We detected no alterations of NF2 gene expression in the quantitative analyses comprising 5 GIST. However, NF2 gene mutations were identified in 2 GIST cases in a study by Fukasawa *et al.*<sup>33</sup>, suggesting that NF2 may act as a tumor suppressor in a subset of these tumors. The NF2 gene mutations detected occurred independently of c-kit mutations, since no correlation between NF2 mutations or LOH at 22q and the c-kit mutations was observed.

Among the aberrant chromosomal regions, the losses of 9p are of particular interest. Homozygous deletions were found in 2 GIST cases in the restricted area of 9p containing the CDKN2A gene<sup>34</sup>. Since homozygous deletion is one of the known mechanisms for inactivation of a tumor suppressor gene, it may be possible that inactivation of CDKN2A plays a role in tumorigenesis of GIST, at least in some cases. In a recent study, chromosomal changes in GIST were investigated in relation to malignant behavior and the presence of c-kit mutations<sup>35</sup>.

Interestingly, associations with malignancy, c-kit mutations and loss of chromosome 9 have been detected. However, only c-kit mutations have been shown to clearly correlate with malignancy and a poor prognosis and thus have been proposed to have an important role as a prognostic factor<sup>36</sup>.

Several factors contribute to genomic instability, and cells accumulate many different genetic changes during carcinogenesis. The multiple forms of genetic changes may reflect cellular heterogeneity seen within individual tumors and heterogeneity between tumors of the same type. This heterogeneity is reflected as differences in the histological, karyotypic, molecular, physiological and biochemical factors that can be observed by examining different parts of the same tumor. In lung cancers, it has been hypothesized that the heterogeneity of bronchial tumors may be a consequence of chronic exposure to numerous different carcinogens, e.g., carcinogens present in tobacco, which leads to several cell clones carrying different genetic changes<sup>37</sup>. This may be the case with other tumors also associated with environmental carcinogen exposure.

Interestingly, many of the genetic alterations detected in invasive lung tumors have been demonstrated also in the bronchial epithelium of both current and former

smokers without cancer, indicating that molecular damage is already present in carcinogen-exposed bronchial cells<sup>38-40</sup>. These observations may provide new methods for the early detection of lung cancer. In addition, identification of genetic alterations crucial for the development of lung cancer can be used for cancer prevention and for the development of novel therapies specifically targeted to the disrupted cellular activity<sup>37</sup>. Recent studies have also highlighted the possibility to use genetic alterations as early markers since these are detectable in plasma or serum DNA from cancer patients, including lung cancer patients<sup>37,41-45</sup>.

Understanding the interplay between exposure to specific environmental carcinogens and genetic variability of important genes will be important for the ultimate goal of comprehending cancer etiology. This new knowledge will help in the identification of individuals who are at increased risk of developing cancer and will hopefully lead to better biomarkers for the early detection of cancer and cancer prevention. Frequent LOH detected at 22q in GIST, especially in malignant tumors, suggests that a tumor suppressor gene located at this chromosomal region is involved in the tumorigenesis of GIST. However, the absence of NF2 gene mutations and the presence of merlin in the tumor tissue are evidence that another, still to be identified gene is responsible for the development of GIST.

In conclusion, even though studies on larger and homogeneous series are certainly necessary, this research can be considered very preliminary to study NF2 expression in GIST.

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