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Influence of 17q gain and promoter polymorphisms on mRNA expression of somatostatin receptor type 2 in neuroblastoma

Lisa Simi [1], Pamela Pinzani [1], Claudia Casini Raggi, Mario Pazzagli, Claudio Orlando *

Clinical Biochemistry Units, Department of Clinical Physiopathology, University of Florence, Italy

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Abstract

Background: Neuroblastoma, the most frequent solid extracranial tumor in children, is characterized by a wide spectrum of clinical behaviours. We previously reported that high expression of somatostatin receptor type-2 (sst2) mRNA is associated to increased overall and event free survival. Several genetic abnormalities are detected in neuroblastomas, frequently involving balanced and/or unbalanced gain on the long arm on chromosome 17, the same region containing sst2 gene.
Methods: In this study we detected balanced and/or unbalanced 17q gain in 50 neuroblastomas. Since two polymorphisms in sst2 promoter (−57 C>G and −83 A>G) were previously described as responsible for an *in vitro* reduction of sst2 mRNA expression, promoter sequencing was also performed in the same samples. The results were compared to sst2 mRNA expression, measured by real-time RT-PCR.
Results: The frequency of 17q gain (14/50 neuroblastomas) was significantly associated to sst2 mRNA over-expression (Fischer's exact test: $p=0.0012$). The sst2 expression was significant higher both in balance and unbalance 17q amplifications (ANOVA: $p=0.04$). Conversely, we found a reduction of sst2 mRNA in neuroblastomas with −57 C>G promoter polymorphism (ANOVA: $p=0.03$).
Conclusion: We highlighted that 17q gain and promoter polymorphisms can play a role into the regulation of sst2 expression in neuroblastomas.

Keywords: Balanced and unbalanced 17q gain; Multiplex real-time RT-PCR; Prognosis

1. Introduction

Neuroblastoma is the most common solid extracranial neoplasm in children and involves sympathetic nervous system consisting of undifferentiated neural crest derived from neuroectodermal cells. This tumor is characterized by different clinical behaviours and clinical phenotypes, defined by a complex pattern of genetic abnormalities [1]. Karyotypes of this tumor frequently include many marker chromosomes and unidentified products of unbalanced translocation, especially in advanced stage of disease. Several genetic aberrations in neuroblastoma have been described to be associated with poor prognosis and their identification is important for patient stratification, to select groups with high risk-level and then adequate therapy [2].

The presence of structural abnormalities correlates with an aggressive phenotype and frequently involves chromosomes 1p, 3p, 11q, 14q, 17q and MYCN amplification [2–4]. In particular, rearrangements of chromosome 17, which were first described in 1980s [5], seem to be a feature of considerable biological significance. The prognostic relevance of 17q gain has been confirmed by several authors [2,6,7]. It has been observed that extra copies of the distal part of the long arm of chromosome 17 (17q gain) is the most frequent abnormality in neuroblastoma cells, with an incidence ranging from 63 to 83% [7]. The role of this genomic alteration in the progression of neuroblastoma is still unclear. Some authors have found that the amplification of the q-arm of chromosome 17, often resulting from an unbalanced translocation der(1)t(1p;17q), is associated with unfavourable prognosis in neuroblastoma suggesting that this

* Corresponding author. Clinical Biochemistry Units, Department of Clinical Physiopathology, Viale Pieraccini 6 50139 Florence, Italy. Tel.: +39 055 4271440; fax: +39 055 4271413.
E-mail addresses: lisa.simi@dfc.unifi.it (L. Simi), p.pinzani@dfc.unifi.it (P. Pinzani), claudiafallani@virgilio.it (C.C. Raggi), m.pazzagli@dfc.unifi.it (M. Pazzagli), c.orlando@dfc.unifi.it (C. Orlando).
[1] These authors contributed equally to this work.

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region includes a gene, or genes, of great biological importance in tumor progression, in particular in stage 4 of the disease [8]. A previous study, performed in a large series of neuroblastomas, clarified the clinical significance of chromosome 17 amplification [4]. In particular, the presence of balanced gain seems predictive of a good patient outcome. Conversely, normal and partial amplification of 17q are associated to worst prognosis, particularly in patients with low-stage tumors.

We previously reported a study on the prognostic role of sst2 mRNA expression in neuroblastoma patients. Higher sst2 mRNA expression was related to positive overall and event free survival, suggesting sst2 mRNA expression as a valid factor to predict outcome, independently from MYCN amplification [9–11]. Nevertheless we observed that a group of patients evidenced a worst prognosis, despite high sst2 mRNA expression levels [9]. Antitumoral properties of sst2 have been confirmed by several works. Somatostatin and its stable analogues are able to suppress proliferation in normal and tumor cells expressing somatostatin receptor [12]. Although no mutations on sst2 gene (17q24) have been found in neuroblastoma [6], two polymorphisms of the sst2 promoter sequence (−83 A>G and −57 C>G from the transcription initiation site) have been described as negative transcription regulators in pancreas cancer [13].

In the present study, we evaluated sst2 mRNA expression in relation to 17q gain and sst2 promoter polymorphisms. Balanced and unbalanced 17q gain were determined simultaneously by a novel triplex real-time PCR method.

2. Materials and methods

2.1. Tissues samples

The study group consisted of 50 (28 male and 22 female; mean age: 31 months; age range from 2 weeks to 198 months) neuroblastoma samples obtained from the Italian Neuroblastoma Tissue Bank (Genoa, Italy). Clinicopathological features of patients were assessed according to the International Neuroblastoma Staging System (INSS) as follows: 11 patients stage I (22%); 7 patients stage II (14%); 5 patients stage III (10%); 20 patients stage IV (40%) and 7 patients stage 4s (14%). Cancer samples obtained from the patients, were snap-frozen in liquid nitrogen; a sample of tumor tissue was processed for routine histological examination. Informed consent of each patients' parents was obtained.

2.2. Quantification of sst2 mRNA expression

For RNA extraction, tissues were disrupted by homogenisation in 600 μl of guanidine isothiocyanate-containing lysis Qiagen buffer (QIAGEN S.p.A., Milan, Italy) added with β-merchaptoethanol. Total RNA was extracted with QIAshredder and RNeasy MiniKit Qiagen® columns. RNA was then eluted from columns with 50 μl of Qiagen® RNase free water. During the extraction process the sample was treated with RNase free DNAse Set QIAGEN® to eliminate DNA. Quantification of sst2 mRNA expression was performed with TaqMan technology as previously reported [9,14]. The same cut-off value (7×10^7 molecules/μg total RNA) previously defined [9] has been used to compare data obtained from genetic analysis and expression pattern of sst2 mRNA.

2.3. DNA sequencing

The presence of polymorphism in the promoter region of sst2 (GenBank Accession number: L34689) was evaluated in the 50 DNA from neuroblastomas and in 88 DNA extracted from whole blood of a normal population. Genomic DNA was extracted from neoplastic tissues using a commercial kit, DNeasy Tissue Kit (Qiagen S.p.A., Milan, Italy). DNA sequencing analysis was performed as reported by Torrisani et al. [13]. Briefly, 100 ng of total DNA were amplified in a PCR reaction mix of 25 μl of final volume. Samples were treated for 9 min at 94 °C and to 35 cycles of amplification at 94 °C for 1 min, 60 °C for 1 min and 72 °C for 90 s. PCR products were purified and submitted to cycle-sequencing reaction: 20 ng of DNA were blended with each primer (0.8 μmol/l) in a Terminator Ready Reaction Mix containing Big Dye Terminators (Applied Biosystems, Italy). Five microliters of marked and purified DNA was submitted to sequencing analysis with ABI PRISM 310 Genetic Analyser (Applied Biosystems, Italy).

2.4. Detection of chromosome 17 status by triplex real-time PCR

Balanced and unbalanced status of 17q have been defined by gene assay of p53 and sst5 genes located in the short arm of chromosomes 17 and 16, respectively. Since genetic abnormalities on p53 and sst5 gene have never been described in neuroblastoma pathogenesis [15,16], these genes were used to evaluate any amplification involving regions in the long arm of chromosome 17.

Sequences of the sst2 and sst5 were obtained from GenBank database. Primers and TaqMan probes were designed using the proprietary software "Primer Express" (PE, Applied Biosystems, Italy). For the amplification of the intron-less gene sst2, primers and probe were the same described for mRNA measurement [9]. Primers and probes selected for sst5 analysis were: forward primer 5′-TCCTCTCCTACGCCAACAGC-3′, reverse primer 5′-GGAAGCT-CTGGCGGAAGTT-3′ and probe 5′ (FAM)-CCCGTCCTCTACGGCTTCCT-CTCTGA-(TAMRA) 3′. Primers and probe for p53 detection were the same as used by T. Tajiri et al. [17].

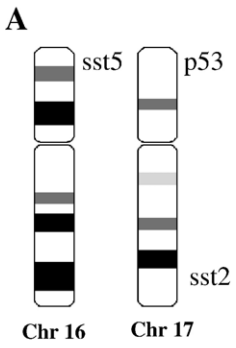
The detection of sst2, sst5 and p53 DNA in each sample was made in triplicate using differently labelled probes to perform a FAM (sst2)/VIC (p53)/TET (sst5) assay in a triplex quantitative real-time PCR method with TaqMan technology. TaqMan PCR was carried out using a reaction volume of 25 μl. One hundred nanograms of DNA were blended in a reaction mix containing 12.5 μl Universal Master Mix 2X (Applied Biosystems, Italy), 200 nmol/l of each fluorescent probe, 300 nmol/l of each primer for sst2 and sst5 detection, 100 nmol/l of p53 primers. The three differently labelled probes were added to the same reaction tube to provide simultaneous measurement of the three genes. Samples were then submitted to 2 min at 50 °C, 10 min at 95 °C and to 40 cycles of amplification at 95 °C for 15 s, 60 °C for 1 min in the ABI Prism 7700 Sequence Detector PE, Applied Biosystems. As negative controls, each run included a duplicate tube evaluation of "no template control" to exclude assay contamination. DNA extracted from three neuroblastoma cell lines was used as positive controls: SH-SY5Y, without 17q amplification, and SK-N-AS and LAN-5, both with unbalanced 17q gain [18].

Relationship between sst2, p53 and sst5 copy number was defined by calculating $2^{-\Delta\Delta Ct}$, using a normal DNA as calibrator [19]. The ratios sst2/p53, sst2/sst5 and p53/sst5 were calculated in the samples and in the calibrator, thus defining three parameters related to the unbalanced (sst2/p53, sst2/sst5) and to the balanced (sst2/sst5, p53/sst5) status (see Fig. 1).

To define the cut-off value for 17q gain determination, sst2/p53, sst2/sst5 and p53/sst5 ratios were evaluated in DNA from 20 normal control subjects. The values of $2^{-\Delta\Delta Ct}$ was calculated for each couple of genes. Relative efficiency of each reaction was calculated considering a standard curve generated with a normal DNA, analyzed in a triplex assay (Fig. 1). For each gene, slope value was obtained and used to define reaction efficiency by the formula: $e=10^{(-1/\text{slope})}-1$. Efficiencies were: sst2=1.08, p53=0.83 and sst5=0.85. The respective cut-off values were obtained adding one standard deviation to the mean, to guarantee the maximal specificity of the assays and adjusting to the relative PCR efficiency [20] and were sst2/p53=1.5, sst2/sst5=1.8 and p53/sst5=1.9.

2.5. Statistical analysis

Statistical analysis was carried out using the SPSS software package (SPSS INC, Chicago, IL). Statistical differences between groups were assessed using Fisher's exact test and ANOVA. Differences with $p<0.05$ were considered statistically significant. To compare statistical difference between unbalanced



17 q gain	sst2/p53	sst2/sst5	p53/sst5	No of patients
No gain	<1.5	<1.8	<1.9	36 (72%)
Unbalanced	≥1.5	≥1.8	<1.9	9 (18%)
Balanced	<1.5	≥1.8	≥1.9	5 (10%)

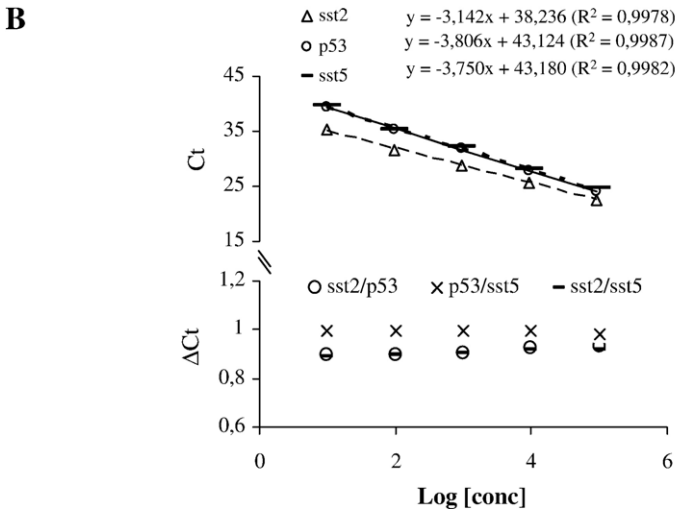


Fig. 1. Measurement of 17q copy number by triplex assay. A: Relationship between sst2, p53 and sst5 copy number and relative cut-off values adopted to evaluate 17q unbalanced and balanced gain. Results obtained in 50 neuroblastoma are also reported. B: Linearity of the assays: Ct and ΔCt values are compared to the concentration of the starting quantity of DNA in each gene measured by triplex assay.

and balanced 17q gain Students' *T* test has been performed. The probability of cumulative survival in various subgroups was tested according to Kaplan–Meier life tables and differences were tested with the log rank method.

3. Results

3.1. Reliability of the triplex real-time PCR assay

To assess linearity of triplex assay, DNA extracted from neuroblastoma cell lines SK-N-AS was serially diluted starting from 100 ng to 10 pg and submitted to real-time PCR for the three genes. As reported in Fig. 1, genes demonstrated a high parallelism, independently from the concentration of the starting material. Moreover, when considering the ΔCt of each pair of genes for each DNA concentration, we always obtained lines whose slope was as close as possible to zero [19] (y=00105x+0.8795 for sst2/p53; y=00078x+08807 for sst2/sst5; y=−00029x+1001 for p53/sst5).

The inter-assay reproducibility was evaluated in five triplex assays, analyzing Ct values obtained from two controls: one DNA from normal sample and one from LAN-5 cell line, as reference of unbalanced 17q gain. Inter-assay reproducibility calculated in the normal sample and in LAN-5 DNA was respectively 2.31% and 4.18% (CV%) for sst2; 4.92% and 3.84% for p53, 3.73% and 5.35% for sst5. Intra-assay reproducibility was calculated from six replicates of DNA from SK-N-AS samples; CV% values were: 0.79% for sst2, 1.84% for p53 and 2.09% for sst5.

3.2. 17q gain in neuroblastomas and absolute sst2 mRNA expression

In 36/50 patients (72%), the sst2/p53, sst2/sst5 and p53/sst5 ratios were simultaneously in the range of normal controls, indicating the absence of chromosome 17 abnormalities. In 9/50 neuroblastomas (18%) we found that sst2/p53 and sst2/sst5 ratios were simultaneously increased over the threshold (Fig. 1). The presence, in the same patients, of a normal p53/sst5 ratio indicated an amplification restricted to the long arm of chromosome 17 and, therefore, an unbalanced gain. The remaining 5 patients (10%) showed the amplification extended to both arms (or even to the whole) of chromosome 17, as evidenced from the increased sst2/sst5 and p53/sst5 ratios over the threshold, but a normal sst2/p53 ratio.

A significant difference of sst2 mRNA expression was found when our patients were classified on the basis of the absence of 17q gain or of the presence of balanced and unbalanced gain (p=0.04, Fig. 2). In patients without evidence of 17q modifications, sst2 mRNA expression was $5.8\times10^8\pm2.0\times10^8$

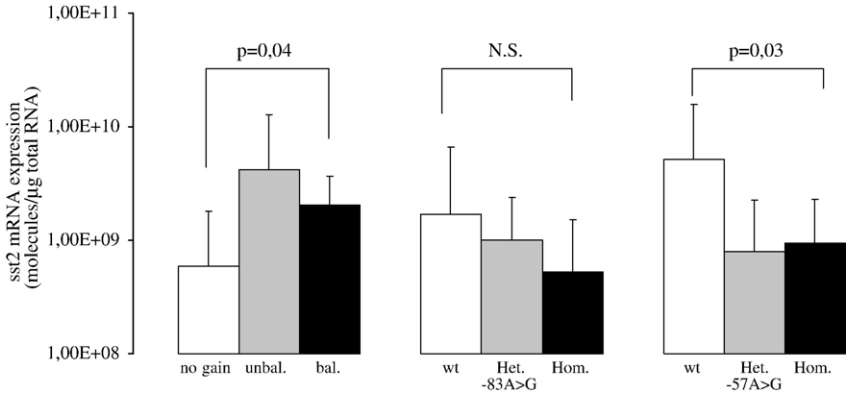


Fig. 2. Expression of sst2 mRNA in 50 NB tumors, compared to 17q gain and to promoter polymorphisms. On the left: absolute values (mean±SD) of sst2 mRNA levels in patients subdivided by 17q gain: no amplification, unbalanced and balanced gain. On the right: absolute values (mean) of sst2 mRNA in patients subdivided by the presence of the promoter polymorphisms: homozygotes for wild type sequence, heterozygotes and homozygotes for −83 and −57 polymorphisms.

molecules/µg total RNA (mean±standard error) whereas in patients with unbalanced and balanced gain, sst2 expression was $4.2\times10^9\pm2.9\times10^9$ and $2.0\times10^9\pm7.4\times10^8$, respectively. This latter difference was not significant. In patients with sst2 mRNA over-expression, unbalanced 17q gain is more frequent (36%) in the group with unfavourable outcome than in those with better prognosis (21%) even if this difference was not statistically significant.

The probability of overall survival according to sst2 expression and 17q amplification, represented as Kaplan–Meier curves, is reported in Fig. 3, confirming that patients with high sst2 expression had a significantly better survival rate (Fig. 3A, $p=0.04$). Considering 17q status we observed a 100% of survival probability in patients with balanced gain, while unbalanced amplification seems to be associated with unfavourable outcome (Fig. 3B). Furthermore, high sst2 mRNA expression levels are predictive of a good prognosis only in patients without 17q aberrations (Fig. 3C). The last two differences were not statistically significant probably due to the low number cases.

3.3. −57 C>G and −83 A>G polymorphisms and absolute sst2 mRNA expression

The −57 C>G polymorphism was detected in 23 subjects as heterozygotes and in 21 as homozygotes (Table 1). In neuroblastomas the allelic frequency of −57 (35% C and 65% G) and −83 polymorphisms (76% A and 24% G) was similar to those observed in our control population (40.2% C and 59.8% G and 70.5% A and 29.5% G, respectively). However, we found that the presence of sst2 promoter polymorphisms was associated to a reduction of sst2 mRNA expression, both for −83 and −57 variants. The level of sst2 mRNA expression in wild type patients for −83 polymorphism (independently from 17q status) was $1.7\times10^9\pm9.1\times10^8$, while in heterozygotes was $1.0\times10^9\pm3.5\times10^8$ and in homozygotes $5.2\times10^8\pm4.9\times10^8$. In the case of −57 polymorphism, in wild type subjects sst2 expression was $5.2\times10^9\pm4.4\times10^9$, significantly higher ($p=0.03$) in comparison to heterozygotes and homozygotes ($7.9\times10^8\pm3.03\times10^8$ and $9.4\times10^8\pm2.94\times10^8$, respectively) (Fig. 2).

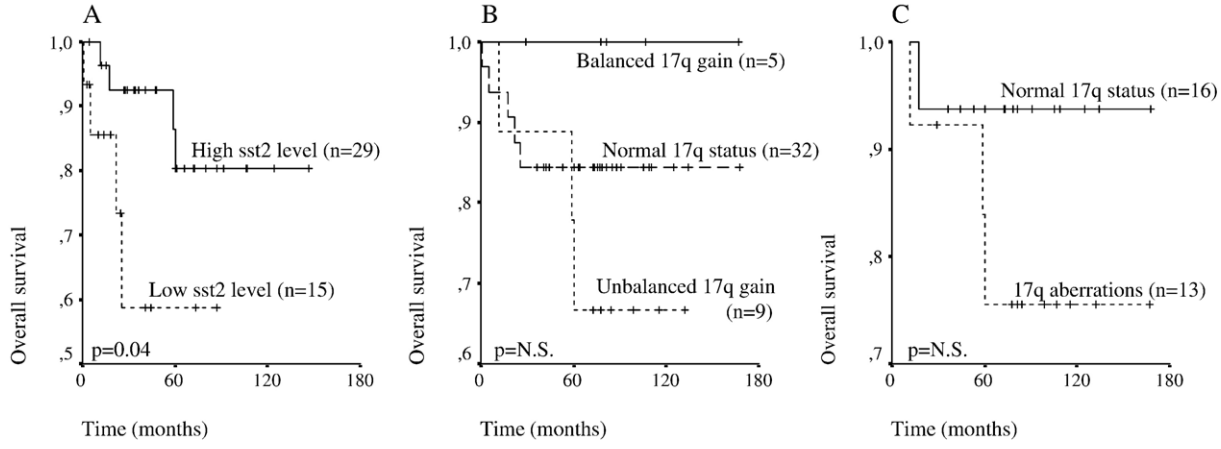


Fig. 3. Overall survival probability in 50 patients with neuroblastoma according to sst2 expression (*left panel*) and 17q gain (*central and right panel*). A)The Kaplan–Meier curve shows the probability of survival in two groups of patients classified on the basis of a reported cut-off (7×10^7 molecules/µg RNA). B) Kaplan–Meier curve in patients subdivided on the basis of 17q gain status. C) Kaplan–Meier curve in patients with high sst2 mRNA expression subdivided on the basis of the presence of 17q aberration.

Table 1
Distribution of patients ($n=50$) on the basis of 17q amplification, promoter polymorphism and sst2 mRNA expression

Variables	n	%	sst2 mRNA expression [a]		p [b]
			Low	High	
17q gain					
Not amplified	36	72	19	17	
Unbalanced gain	9	18	1	8	
Balanced gain	5	10	0	5	0.012
sst2 promoter polymorphism [c]					
−57 C>G					
Wild type	6 (16)	12 (18.2)	1	5	
Heterozygotes	23 (39)	46 (44.3)	8	15	
Homozygotes	21 (33)	42 (37.5)	11	10	N.S.
−83 A>G					
Wild type	30 (49)	60 (55.7)	10	20	
Heterozygotes	16 (26)	32 (29.5)	7	9	
Homozygotes	4 (13)	8 (14.8)	3	1	N.S.

[a] Low and high expression of sst2 mRNA is determined considering the cut-off value of 7×10^7 molecules/μg total RNA.
[b] The p value were calculated from Fisher's exact test and is significant when <0.05.
[c] In brackets are reported the results obtained in our control population ($n=88$).

3.4. 17q gain, sst2 promoter polymorphisms and prognosis

As previously reported [9], we classified neuroblastoma patients as expressing high or low sst2 mRNA according to a biological cut-off (7×10^7 molecules/μg RNA). This cut-off was previously determined as the threshold that separated the somatostatin-responsive from unresponsive neuroblastoma cell lines [21]. On this basis, sst2 mRNA expression can discriminate patients with good prognosis (high expression) from those with worse outcome (reduced expression). When our group of neuroblastomas was stratified according to this cut-off, we found that only 1/20 patients with low sst2 mRNA expression had an unbalanced 17q gain, whereas 13/30 patients with high sst2 had balanced or unbalanced gain. It is to note that 6 patients of this latter group had an adverse outcome (alive with disease or dead of disease) in spite of the high sst2 mRNA expression. Among the 20 patients with sst2 levels below the cut-off, 10 of them had unfavourable outcome and none of them had 17q gain.

In our group of neuroblastomas, 10 patients exhibit good prognosis (absence of relapse with a mean follow-up of 73, 4 months) in spite of the low sst2 mRNA expression. In this subset we found a significant increase ($p=0.01$) of −83 polymorphism (80%) in comparison to the patients with expected adverse prognosis (20%).

4. Discussion

Several studies performed in neuroblastoma tumors have identified a common amplified region ranging from 17q21.3 to 17qter with a highly variable localization of the breakpoint region [2,22]. Identification of one or more genes located in the long arm of chromosome 17 is of large clinical interest since an effect of their altered expression may be responsible for the altered phenotype [23]. Most prominent candidate are the anti-apoptotic regulatory genes: nm23-H1, -H2 and survivin. Nm23-H1 and -H2 are members of a large family up regulated by MYCN and c-MYC, thus accumulation of their mRNA levels may be associated with MYCN amplification in tumors with 17q gain [23]. Increased expression of survivin has been found to be correlated significantly with adverse prognosis and outcome [24,25] but direct correlation between extra copy of 17q and gene expression in neuroblastoma has not yet been demonstrated for this gene. Recently, extra copy numbers of 17q have been correlated to WSB1 (17q11.1) gene expression [26].

The statement that the amplification of 17q may be an independent predictor of disease outcome and the high incidence in patients affected by neuroblastoma has reinforced its identification in cytogenetics analysis. A PCR-based approach in the detection of 17q amplification could make the use of this parameter easier in defining tumor characteristic. A validated duplex genomic real-time quantitative PCR assay has been yet reported to evidence unbalanced status in neuroblastoma samples [27] suggesting the use of this method for a rapid screening, but this approach fails to detect balanced gain of 17q, equally responsible of an altered phenotype.

In this study we firstly investigated 17q amplification by using a triplex assay method allowing a rapid determination of both balanced and unbalanced status of chromosome amplification. Further comparison of our results should be performed with those obtained by approved techniques as FISH or CGH before the method here described can be proposed as suitable for diagnostic purposes. Results obtained in this work confirm our previous study where sst2 mRNA measurement was suggested as an independent prognostic marker in neuroblastoma [9], but also evidenced that a low number of subjects did show high sst2 expression despite an advanced stage disease and poor prognosis. Our hypothesis is that this apparent contradiction depends upon genetic aspects, as amplification of 17q, that directly causes the increase of the copy number of sst2 gene, responsible of an altered expression pattern.

Data obtained by comparing 17q gain and sst2 levels seem to confirm an association between the two parameters: 13/14 patients with 17q balanced or unbalanced amplification show also high expression of sst2 mRNA. Even if sst2 mRNA expression cannot be considered as a marker of 17q gain, the amplification of the long arm of this chromosome seems to be strictly related to an increase in its expression.

Interestingly, overall survival analysis showed that the whole chromosome 17q gain is associated with favourable outcome, while unbalanced gain confers poor prognosis, thus confirming previously reported data [4].

Polymorphisms on sst2 promoter at position −83 A>G and −57 C>G of the transcription initiation site were frequently detected in pancreatic carcinoma. The −83 A>G has been found to promote specific fixation of nuclear factor I (NF1) and thus repression of sst2 mRNA transcription [13]. In our study, the presence of sst2 promoter polymorphisms appeared not directly related to neuroblastoma, since it was found in the normal population with almost the same incidence. Nevertheless, a significant

relationship between sequence for −57 variant and reduction in sst2 mRNA levels was demonstrated. Furthermore, in the group with good prognosis, polymorphism −83 is significantly ($p=0.01$) related to low levels of sst2 mRNA expression.

Further study should be performed to evaluate other genetics features which can be involved in sst2 expression in neuroblastoma as hypermethylation of CpG islands, microsatellites instability and chromosomal deletions.

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