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Abstract

The tumor suppressor TP53, promotes nerve growth factor receptor (NTRK1) Y674/Y675-phosphorylation (NTRK1-pY674/pY675), via repression of the NTRK1-phosphatase PTPN6, in a ligand independent manner, resulting in suppression of breast-cancer cell proliferation. Moreover, NTRK1-pY674/pY675 together with low levels of PTPN6 and TP53 expression is associated with favorable disease-free survival of breast-cancer patients. We determined whether in neuroblastoma this protein expression pattern impacts on relapse-free-survival (RFS). NTRK1-pY674/pY675, PTPN6, and TP53 expression was assessed in 98 neuroblastoma samples by immunohistochemistry. Association between expression levels and RFS was investigated by multivariate and Kaplan-Meier analysis. Mutant or wild-type *TP53* was identified by sequencing tumor DNA. Tumors expressing NTRK1-pY674/pY675 and low or undetectable levels of PTPN6 and TP53 were significantly associated with 5-year RFS (P=0.014) when the data-set was stratified by *MYCN*-amplification, segmental chromosomal abnormalities and histology. Similar results were observed with tumors expressing wild-type TP53, NTRK1-pY674/pY675 and low or undetectable levels of PTPN6. Kaplan-Meier analysis demonstrated a significant correlation (P=0.004) with a 50% probability of RFS (median-survival 4.73 years) when present, compared with 19.51% (median-survival 11.63 months) when absent. Similar results were seen with non-amplified *MYCN* or unfavorable/undifferentiating samples and tumors from patients aged 18 months or less. Importantly, NTRK1-pY674/pY675 is an independent predictor of improved RFS. These results strongly suggest that NTRK1-pY674/pY675 together with wild-type TP53 and undetectable or low levels of PTPN6 expression is a potential biomarker of improved RFS of neuroblastoma patients. The predictive value of NTRK1-pY674/pY675 together with wild-type TP53 and low PTPN6 expression could contribute to neuroblastoma patient prognosis.

Key words: NTRK1, PTPN6, TP53, neuroblastoma

1. Introduction

Neuroblastomas are tumors of sympathoadrenergic origin and the most common extracranial childhood tumour representing 6% to 8% of all childhood cancer [1,2]. Almost 90% of cases are diagnosed before 5 years of age with 30% of these arising within 12 months of birth [1,2]. Long-term survival for children with high-risk type of neuroblastoma is poor [1,2]. Prognosis is related to patient age and risk stage [1,2]. Good-prognosis tumours are differentiated, show favorable cytogenetics [1,2] and express high levels of nerve growth factor (NGF) receptor tyrosine kinase NTRK1 [3,4]. Poor-prognosis neuroblastomas are poorly differentiated, show *MYCN* amplification and segmental chromosomal abnormalities (SCA) (such as 17q gain, 1p and 11q loss) [1,2, 5-7] and preferentially express neurotrophin receptor tyrosine kinase NTRK2 [3]. On NGF-stimulation, NTRK1 is activated by tyrosine (Y)-phosphorylation at positions 490, 670, 674, 675 and 785. This induces signalling cascades promoting survival or differentiation of neuroblastoma cells [4].
The tumor-suppressor TP53 promotes apoptosis or cell-cycle arrest by regulating transcription via protein complexes such as NF-Y [8,9]. In neuroblastoma TP53 mutation incidence is 5-15% [10]. Tumors show wild-type TP53 protein located preferentially in the nucleus, suggesting cytoplasmic sequestration as a possible mechanism of abolishing its tumour-suppressor activity [11]. PTPN6 is a tyrosine phosphatase expressed in hematopoietic and non-hematopoietic cells and dephosphorylates receptor tyrosine kinases including c-kit and receptor associated proteins [12,13]. PTPN6 dephosphorylates tyrosines 674 and 675 (Y674/Y675) of NTRK1 [14]. To date the significance of PTPN6 expression in neuroblastoma has not been determined.

NTRK1 and TP53 play vital and complementary roles in proliferation and differentiation of neoplastic cells, thus suggesting a functional connection between them [4,9]. This is supported by our experiments in the rat sympathoadrenergic PC12 cell line, where TP53 overexpression induces NTRK1-activation and tyrosine phosphorylation, and NTRK1-dependent signal transduction, promoting their NGF-independent differentiation [15,16]. In breast-cancer NTRK1 is expressed in malignant cells with high levels associated with good prognosis and increased patient survival [17]. This is also seen in neuroblastoma [3]. The incidence of *TP53* mutation in breast-cancer is 20-30% [10] and as in neuroblastoma, tumors express wild-type TP53 in both the nucleus and cytoplasm [11]. We have revealed that in breast-cancer, increased levels of wild-type TP53 repress PTPN6 expression [18]. PTPN6 repression induces NTRK1-Y674/Y675 phosphorylation (NTRK1-pY674/pY675), leading to NGF-independent NTRK1-activation [18]. This signals suppression of breast-cancer cell proliferation via cell-cycle arrest [18]. We have also shown that, together, expression of NTRK1-pY674/pY675, wild-type TP53 and undetectable levels of PTPN6 is associated with improved 15-year disease-free survival of breast-cancer patients [19].

Neuroblastomas are biologically heterogeneous [1,2] making it difficult to identify useful biomarkers that predict patient outcome. Breast-cancer and neuroblastomas share similar

characteristics regarding NTRK1 and TP53 as both are involved in cell-cycle control and differentiation [3,4,9]. Given that ganglionic cell differentiation is a favorable prognostic factor for neuroblastoma [1,2], we have investigated whether the presence of wild-type TP53 and PTPN6 protein expression or downregulation together with NTRK1-pY674/pY675 (which are components of the NGF-independent mechanism for NTRK-Y674/Y675 phosphorylation [18]), is predictive of outcome in neuroblastoma tumours. We hypothesised that tumors expressing phosphorylated NTRK1-Y674/675 and wild-type TP53 but with low or undetectable levels of PTPN6 are significantly associated with favourable 5-year relapse-free survival (RFS). To investigate this hypothesis, the levels of NTRK1-pY674/pY675, NTRK1, PTPN6 and TP53 protein expression were assessed in 98 neuroblastoma samples, using immunohistochemistry, and correlated with 5-year RFS. Multivariate analysis demonstrated that moderate and strong levels of NTRK1-pY674/p675 together with undetectable levels of PTPN6 and TP53 correlate with increased RFS. Similar results were observed with samples encoding wild-type TP53, non-amplified *MYCN* and with unfavorable/undifferentiating samples. Furthermore, this protein pattern remained an independent predictor of outcome regardless of patient age. Significantly, the presence of NTRK1-pY674/pY675 alone was an independent predictor of 5-year RFS by multivariate analysis when parameters were adjusted for *MYCN* amplification, SCA and histology. Although it is documented that NTRK1 expression is associated with favorable patient survival [3], our results emphasise the importance of functional NTRK1 as an indicator of neuroblastoma good prognosis. Overexpression of PTPN6 and TP53 was associated with shorter RFS. These findings strongly suggest that expression of NTRK1-pY674/pY675, together with wild-type TP53, and undetectable levels of PTPN6, correlate with improved RFS and favorable outcome of neuroblastoma patients.

2. Materials and Methods

2.1 Tumor samples and DNA

TP53, PTPN6, non-phosphorylated NTRK1 or NTRK1-pY674/pY675 expression was assessed in 98 formalin fixed paraffin-embedded neuroblastomas samples arranged in tissue microarrays. Each tissue microarray block contained 15 to 20 two millimetre diameter cores. Sample clinicopathological data was available (Department of Histopathology, Great Ormond Street Hospital, UK). This included presence of *MYCN* amplification and segmental chromosomal abnormalities (SCA) status. Genomic DNA from 60 matched tumors was extracted using standard protocols. Approval was obtained from the NHS Research Ethics Committee (REF 11/LO/1468).

2.2 Immunohistochemistry

Tissue microarray sections were dewaxed in xylene and rehydrated. A Pascal pressure heating chamber was used for antigen retrieval with Dako Citrate Buffer (pH 6.2) for 2 min. Sections were incubated for 40 min at room temperature with TP53 PAb DO-7 monoclonal antibody 1:100 dilution, Dako, Denmark; PTPN6 polyclonal antibodies 1:1000 dilution, Santa Cruz Biotechnology,

USA; NTRK1 Trk-B3 monoclonal antibody 1:200 dilution, Santa Cruz Biotechnology, USA; and polyclonal antibodies against NTRK1-pY674/pY675 1:150 dilution, ABgent, USA. Sections were rinsed with PBS and incubated with blocking substrate buffer (Dako, Denmark). After two rinses, bound antibody was detected using the Envision/HRP kit (Dako, Denmark). Then were rinsed in tap water and counterstained with hematoxilin. Sections were also stained using the Ventana BenchMark ULTRA IHC/ISH staining platform with the mentioned antibody dilutions. Positive and negative controls were included. Sections were scanned on a Hamamatsu Nanozoomer S360 Digital Slide Scanner and viewed using NDP.view2. Staining was undertaken in triplicate non-consecutive sections.

2.3 Genomic DNA Sequencing

DNA was subjected to PCR to amplify exons 5 to 9 of the TP53 gene by using the Tetrad 2 Peltier Thermal Cycler (Biorad, UK). Primers used: exon 5 forward (5 'ACGTGTGCCCTGACTTTCAAC T 3') and reverse (5' CAATCAGTGAGGAATCAGAGGC 3'). Exon 6 forward (5' TCAGATAGCGATGGTGAGCAG 3') and reverse (5' GCCACTGACAACCACCCTTA 3'). Exon 7 forward (5' AGGCGCACTGGCCTCATCTT 3') and reverse (5' GAAATCGGTAAGAGGTGGGC 3'). Exon 8 forward (5' GGAGTAGATGGAGCCTGGTTT 3') and reverse (5' GGTGATAAAAGTGAATCTGAGGC 3'). Exon 9 forward (5' GGAGACCAAGGGTGCAGTTAT 3') and reverse (5' GTTAGTTAGCTACAACCAGGAGCC 3'). Conditions for the reaction steps were an initial step of 94^0C for 5 min followed by 35 cycles comprising of 94^0C for 1 min for denaturation, 60^0C for 1 min for annealing, 11 min at 72^0C for elongation. Amplicons were prepared for sequencing using the BigDye Terminator Cycle Sequencing Kit. The products were run on an ABI automated sequencer (Applied Biosystems, CA, USA). Both DNA strands were sequenced. Mutation presence was confirmed by repeating the analysis. Sequence traces were analysed using Mutation Surveyor Software v3.20 (SoftGenetics, PA, USA).

2.4 Statistical Analysis

TP53, PTPN6, NTRK1, and NTRK1 at pY674/pY675 protein expression levels were assessed using a semi-quantitative intensity score whereby absent, weak, moderate, or strong was denoted as 0, 1, 2, and 3, and based on the methods of Dowsett *et al.* and Pinder *et al.* [20,21]. 50% or more positive neuroblast cells within a core was the criteria to denote expression intensity, otherwise it was considered negative. Scoring (blinded to tumor type), was independently undertaken by three of the authors (GY, XM and CG). Inter-observer variability was resolved by discussion between these authors.

The dataset was stratified by presence or absence of *MYCN* amplification, SCA (1p and 11q deletion, and 17q gain), presence of wild-type *TP53*, favorable/differentiating or unfavorable/undifferentiating histology and age (less than or older than 18 months).

Analysis for 5-year relapse-free survival (RFS) was carried out in order to determine the prognostic significance of tumors expressing NTRK1-pY674/pY675 together with undetectable or low levels of TP53 and PTPN6. RFS is defined as the time of diagnosis until relapse or death. The Kaplan-Meier method was used to calculate estimates of cumulative survival probabilities (survival function) for this protein pattern. Kaplan-Meier plots were generated and the Log-rank statistic was used to determine the statistical significance of the differences between the survival functions. A Cox regression hazard model was generated and multivariate analysis was performed. For all statistical tests, the significance threshold was taken at 95% confidence interval and a P =0.05 or less. Linear regression was performed to determine the predictive value of the expression pattern in accounting for the variances in 5-year relapse-free survival times. SPSS (version 25) and STATA software (release 14.0) were used for statistical analysis.

3. Results

3.1 Clinicopathological characteristics and outcome of patients

The clinical and pathological features of the 98 patient neuroblastoma samples are shown in Supplementary Table S1. The mean age from birth until date of diagnosis was 36.98 months (standard deviation=57.77 months) with a range of 0.024 to 170.40 months. Age at time of diagnosis is critical on survival outcomes, as children diagnosed at 18 months or less have a better prognosis than those diagnosed at an older age [1,2, 22]. Therefore, patients were grouped as younger than 18 months (N=41, 41.8%) and older than 18 months (N=53, 54.1%). Age at diagnosis information was missing for four patients.

The presence of amplified *MYCN* and SCA correlates with poor survival and response to treatment of high-risk neuroblastoma patients [5-6, 23]. Amplified and non-amplified *MYCN* was detected in 15 tumors (15.3%) and 69 tumors (70.4%), respectively. Presence of SCA were seen in 37 tumors (1p deletion (N=14, 14.3%), 11q deletion (N=9, 9.2%) and 17q gain (N=28, 26.6%)). Some tumors had more than one single chromosomal abnormality. Histological subtype was considered as favorable/differentiating and unfavorable/undifferentiating morphological states are associated with good and poor outcome, respectively [1,2]. The sample cohort comprises of 18 differentiating neuroblastomas, 18 intermixed ganglioneuroblastomas, 2 ganglioneuromas, 1 nodular ganglioneuroblastoma and 59 undifferentiating neuroblastomas. Histology was reviewed by Professor Neil Sebire, one of the authors.

3.2 Expression of NTRK1-pY674/p675, NTRK1, PTPN6 and TP53 in neuroblastoma tumors

Antibodies and immunohistochemical techniques used to assess the presence of NTRK1-pY674/pY675, NTRK1, PTPN6, and TP53 in formaldehyde-fixed and paraffin-embedded tissue are well recognized and specific [19,24-27]. The frequencies of expression intensities for NTRK1-pY674/pY675, NTRK1, PTPN6, TP53 are summarized in Supplementary Table S2.

We have revealed that NTRK1-pY674/pY675 is an independent prognostic indicator of improved disease-free survival in breast-cancer patients [19]. Thus, the presence of non-phosphorylated NTRK1 and phosphorylated NTRK1-pY674/pY675 was determined in neuroblastoma samples. NTRK1 was expressed preferentially in the cytoplasm of tumor cells (Figure 1). The punctuate pattern is consistent with reports demonstrating the internalisation of membranous NTRK1 during retrograde transport towards the cell bodies of neurons [25]. Weak, moderate and strong expression of NTRK1 was present in 19 (19.4%), 31 (31.6%) and 27 (27.6%) samples, respectively. In 21 (21.4%) cases, NTRK1 was undetectable (Supplementary Table S2). NTRK1-pY674/pY675 presence was seen mostly in the membrane as previously described [25] (Figure 1). Weak, moderate and strong levels of phosphorylation were detected in 33 (33.7%), 27 (27.6%) and 10 (10.2%) tumours, respectively. NTRK1-pY674/pY675 was undetectable in 27 samples (27.6%) and was not determined in one sample due to a missing core (Supplementary Table S2). PTPN6 was localised in the nucleus and cytoplasm (Figure 1) as previously observed in head and neck, and cervical malignant tumors [12,26]. Weak levels were seen in 28 (28.6%) samples. Moderate or strong levels were present in 7 (7.1%) and 4 (4.1%) tumors, respectively. PTPN6 was undetectable in 59 samples (60.2%) (Supplementary Table S2). TP53 was preferentially seen in the nucleus as reported in favourable/differentiating and unfavourable/undifferentiating neuroblastoma [28] (Figure 1). Weak, moderate or strong expression was seen in in 25 (25.5%), 10 (10.2%) and 6 (6.1%) tumors respectively. TP53 was undetectable in 57 samples (58.2%) (Supplementary Table S2).

3.3 Expression of NTRK1-pY674/pY675, NTRK1, PTPN6 and TP53 in neuroblastoma and their association with relapse-free survival

To assess whether expression of NTRK1-pY674/pY675, NTRK1 and low or undetectable levels of PTPN6 and TP53 could have prognostic significance in neuroblastoma, a multivariate Cox Proportional Hazard model adjusted for *MYCN* amplification, SCA and histology type was undertaken for 5-year RFS (Table 1). Patients whose tumors expressed moderate and high NTRK1-pY674/pY675 levels, independent of NTRK1 expression, showed significantly decreased risk of relapse (60%) (hazard ratio=0.40, *P*=0.006). Samples with strong and moderate NTRK1 expression, independent of NTRK1-pY674/pY675, were significantly associated with improved RFS (74%) (hazard ratio=0.26, *P*=0.038) (Table1). Samples expressing both moderate and strong PTPN6 and TP53 were associated with a trend towards reduced 5-year RFS with hazard ratios of 1.69 (*P*=0.204) and 1.55 (*P*=0.265), respectively. These results suggest that NTRK1-pY674/pY675 and NTRK1 presence is significantly associated with prolonged RFS, whereas overexpression of PTPN6 and TP53 appears to be linked to reduced RFS. These variables remained significant in the presence of amplified *MYCN* an established poor outcome prognostic indicator [5-7,23]. Amplified *MYCN* was significantly associated with reduced 5-year RFS with a hazard ratio of 3.68 (*P*=0.002). These results suggest that strong and moderate expression

of phosphorylated NTRK1-Y674/Y675 and NTRK1 are independently associated with 5-year RFS regardless of whether amplified *MYCN* and SCA are included in the analysis (Table 1).

When NTRK1 expression was removed from the Cox Hazards Model (Supplementary Table S3), strong and moderate levels of NTRK1-pY674/pY675 retained prognostic significance, as they were significantly associated with improved relapse-free survival (58%) (hazard ratio=0.42, *P*=0.020). Similar results were seen when NTRK1-pY674/pY675 was removed from the analysis and NTRK1 was assessed, as results showed improved 5-year RFS (59%) (hazard ratio=0.41, *P*=0.021) (Supplementary Table S4). Although it has been established that NTRK1 presence is a good prognosis indicator [3], these results emphasize the relevance of functional NTRK1 (NTRK1-pY674/pY675) as an indicator of prolonged neuroblastoma RFS.

Wild type *TP53* is expressed at low or undetectable levels in cancer cells [27]. To determine whether NTRK1-pY674/pY675 together with undetectable or low levels of PTPN6 and TP53 expression is associated with RFS, tumors showing this combination of NTRK1-pY674/pY675, PTPN6 and TP53 expression were grouped and analyzed as they meet the condition described by Montano [18]. Tumors with TP53 and PTPN6 scores of 0 and 1, and NTRK1-pY674/pY675 score of 2 and 3 as well as samples with TP53 and PTPN6 scores of 0 and NTRK1-pY674/pY675 score of 1 were classed as Group 1. PTPN6 and TP53 scores of 1, 2 and 3 and NTRK1-pY674/pY675 score of 0 were classed as Group 0. From 98 samples, Group 1 and Group 0 expression was present in 37 (36.3%) and 12 (12.2%) tumors, respectively. Multivariate analysis of 49 tumors (Table 2) showed Group 1 expression significantly and independently predictive of 5-year RFS (64%) (hazard ratio=0.36, *P*=0.014) in the presence of *MYCN* amplification (hazard ratio=4.78, *P*=<0.001) (Table 2). These results are supported by the Kaplan-Meier survival analysis showing a significant separation in survival outcome between Group 1 and Group 0 patients (Figure 2). It was estimated that patients with Group 1 tumors had 50% probability of 5-year RFS with a median survival time of 4.73 years, whereas in its absence, survival was reduced to 19.51% with a median time to relapse of 11.63 months (*P*=0.004).

In order to explore further the association of phosphorylated NTRK1-Y674/Y675 together with undetectable or low levels of PTPN6 and TP53, the strength of the relationship between this pattern and 5-year RFS was analysed by linear regression (Predictor variable=pattern, Response variable=5-year relapse-free survival). Results showed that Group 1 positively correlates with improved outcome (R=0.468) and explains 21.88% of the variation in 5-year RFS [$F(1,43)=24.65$, $p<0.001$)]. Together, these results strongly suggest that NTRK1-pY674/pY675 together with undetectable or low levels of PTPN6 and TP53 is associated with favorable survival outcome and is an independent predictor of prolonged 5-year RFS of neuroblastoma patients.

3.4 Expression of NTRK1-pY674/pY675, NTRK1, and PTPN6 in wild-type *TP53* encoding neuroblastoma tumors and their association with relapse-free survival

Since immunohistochemistry does not differentiate between mutant and wild-type *TP53,* the genotypic status of *TP53* was determined by DNA genomic sequencing of exons 5-9. These were chosen as they undergo high mutation frequency in cancer [10]. DNA sequencing was carried out in PCR-amplified regions from genomic DNA available from 60 tumors. Wild-type and mutant *TP53* was present in 52 (86%) and 8 (13%) samples, respectively. This is consistent with studies reporting mutation frequencies of 5–15% in neuroblastoma [10].

Multivariate analysis for 5-year RFS revealed that moderate and high expression of NTRK1-pY674/pY675 was significantly associated with improved survival (72%) (hazard ratio=0.28, *P*=0.005) (Supplementary Table S5). Samples with moderate and high levels of NTRK1 were significantly associated with improved RFS (56%) (hazard ratio=0.44, *P*=0.040). Moderate and high levels of PTPN6 expression showed a trend towards reduced RFS with a hazard ratio of 2.41 (*P*=0.112). As expected *MYCN* amplification and SCA were significantly associated with 5-year RFS with hazard ratios of 4.70 (*P*<0.001) and 3.97 (*P*=0.013), respectively (Supplementary Table S5). These findings suggest that wild-type TP53 expression is associated with NTRK1-pY674/pY675, NTRK1 and PTPN6 similarly to the analysis performed in the whole database shown in Table 1.

In order to explore whether tumours expressing wild-type TP53 together with NTRK1-pY674/pY675 and undetectable or low PTPN6 expression are associated with 5-year RFS, multivariate analysis of 52 tumors expressing this pattern was carried out (Table 3). From 52 samples, Group 1 was present in 35 (67%) and absent in 17 (33%) tumors. Results revealed significant association of Group 1 with 5-year RFS (65%) (hazard ratio=0.35, *P*=0.010) when *MYCN* amplification, SCA and histology status was included in the analysis. *MYCN* amplification was significantly associated with reduced RFS with a hazard ratio of 3.50 (*P*=0.004). Kaplan-Meier analysis showed a significant separation in survival between patients with tumors presenting Group 1 expression with a 48.48% probability of 5-year RFS and a median survival time of 4.9 years compared when absent with a 23.53% probability of 5-year RFS with a median survival time of 11.63 months (*P*=0.042) (Supplementary Figure S1A). These results strengthen the favorable prognostic significance of wild-type TP53 and undetectable or low PTPN6 levels together with NTRK1-pY674/pY675 for RFS in neuroblastoma patients.

3.5 NTRK1-pY674/pY675, NTRK1, PTPN6 and TP53 in *MYCN* non-amplified tumors, unfavorable/undifferentiating samples and at age of diagnosis

Given the importance of the presence or absence of amplified *MYCN* in patient survival [1,2,7], multivariate analysis was performed to determine the prognostic significance of NTRK1-

pY674/pY675, NTRK1, PTPN6 and TP53 as independent predictive parameters in patients with tumors lacking *MYCN* amplification. Analysis of 69 samples with non-amplified *MYCN* revealed that moderate and high expression of NTRK1-pY674/pY675 was significantly associated with 5-year RFS (75%) (hazard ratio=0.25, *P*=0.008) and NTRK1 expression was associated with a trend towards increased RFS (54%) (hazard ratio=0.46, *P*=0.154) (Supplementary Table S6). The presence of SCA was significantly associated with reduced 5-year RFS with hazard ratio of 3.02 (*P*=0.045) (Supplementary Table S6). Analysis for NTRK1-pY674/pY675 together with undetectable or low PTPN6 and TP53 expression showed Group 1 expression present in 30 (52%) and absent in 28 (48%) tumors, respectively. Multivariate analysis revealed significant association of Group 1 expression with 5-year RFS (67%) (hazard ratio=0.33, *P*=0.001) (Table 4).

Given the low number of samples encoding amplified *MYCN* (N=15), a univariate analysis for the expression of phosphorylated NTRK1-Y674/Y675 was undertaken. The presence of NTRK1-pY674/pY675 trended towards favorable 5-year RFS by 34% (hazard ratio=0.66). However, the *P* value was higher than 0.05 (*P*=0.157) (Supplementary table S7). This result suggests that the presence of NTRK1-pY674/pY675 could be a possible indicator of prolonged RFS in patients with *MYCN* amplified tumors.

Historically, neuroblastomas have been classified as favorable/differentiating (with ganglionic characteristics) or unfavorable/undifferentiating (with neuroblastic characteristics) [1,2]. The dataset comprises of 38 (38.8%) favorable/differentiating and 60 (61.2%) unfavorable/undifferentiating tumors. As undifferentiating tumors are linked to poor survival [1,2], multivariate analysis for the presence of NTRK1-pY674/pY675, NTRK1, PTPN6 and TP53 was undertaken to assess their predictive power (Supplementary Table S8). Moderate and high expression of NTRK1-pY674/pY675 was significantly associated with extended RFS (81%) (hazard ratio=0.19, *P*=0.042). Importantly, moderate and high expression of PTPN6 was significantly associated with reduced 5-year RFS with a hazard ratio of 4.03 (*P*=0.005). Moderate and high TP53 expression showed a trend towards reduced RFS (hazard ratio of 2.17, *P*=0.152) (Supplementary Table S8). Analysis of unfavorable/undifferentiating samples for NTRK1-pY674/pY675 together with undetectable or low PTPN6 and TP53 expression showed Group 1 expression present in 14 (47%) and absent in 16 (53%) tumors. Multivariate analysis revealed significant association of Group 1 expression with improved 5-year RFS (66%) (hazard ratio=0.34, *P*=0.007) (Table 5).

As age at time of diagnosis has an effect on patient outcome [1,2], multivariate analysis was undertaken to determine the RFS predictive power of NTRK1-pY674/pY675, NTRK1, PTPN6 and TP53 in patients younger and older than 18 months since this is a cut off at which age impacts on survival in clinical studies [2]. Analysis of 41 tumors from patients younger than 18 months

demonstrated that moderate and high NTRK1-pY674/pY675 expression was significantly associated with prolonged 5-year RFS by (83%) (hazard ratio=0.17, *P*=0.039) (Supplementary Table S9). The presence of *MYCN* amplification was significantly associated with reduced RFS with a hazard ratio of 9.35 (*P*=<0.001). Expression of NTRK1-pY674/pY675 and undetectable or low PTPN6 and TP53 expression (Group 1) was present in 19 (51%) and absent in 18 (49%) samples. Results showed that NTRK1-pY674/pY675 and undetectable or low PTPN6 and TP53 expression was significantly associated with improved RFS (78%) (hazard ratio=0.22, *P*=0.007) (Table 6).

For patients older than 18 months, the analysis followed a similar trend as those diagnosed when younger (Supplementary Table S10). Moderate and high NTRK1-pY674/pY675 expression was associated with a trend towards improved 5-year RFS (69%) (hazard ratio=0.31, *P*=0.093). Moderate and high levels of NTRK1 were significantly associated with favourable RFS (82%) (hazard ratio=0.18, *P*=0.05). Moderate and high TP53 expression, and *MYCN* amplification were associated with a trend towards reduced 5-year RFS with a hazard ratios of 3.77 (*P*=0.276) and 2.60 (*P*=0.351), respectively (Supplementary Table S10). Expression of NTRK1-pY674/pY675 together with undetectable or low PTPN6 and TP53 (Group 1) was present in 16 (38%) and absent in 26 (62%) tumors. Results showed that Group 1 protein expression is associated with a trend towards prolonged 5-years RFS (50%) (hazard ratio=0.50, *P*=0.135). *MYCN* amplification was significantly associated with reduced RFS with a hazard ratio of 3.67 (*P*=0.002) (Supplementary Table S11).

These results suggest that expression of NTRK1-pY674/pY675 alone is an independent predictor of improved survival for patients with *MYCN* non-amplified or unfavorable/undifferentiating tumors, and for patients younger and older than 18 months of age. Importantly, NTRK1-pY674/pY675 together with undetectable or low PTPN6 and TP53 expression provides an independent prediction factor of survival outcome as it is associated with improved RFS for these patient subgroups.

4. Discussion

Results from this investigation of a cohort of 98 neuroblastoma tumors, strongly suggest that NTRK1-pY674/pY675 expression together with undetectable or low levels of expression of PTPN6 and TP53 (Group 1) is independently and significantly associated with prolonged 5-year RFS when the data set was stratified by *MYCN* amplification, SCA and histology status. Similar findings were seen during analysis of non-amplified *MYCN* and unfavorable/undifferentiated tumor groups, and tumors from patients younger than 18 months of age.

Importantly, we have demonstrated that NTRK1-pY674/pY675 alone was significantly and independently predictive of prolonged RFS in a multivariate analysis that included NTRK1, PTPN6 and TP53, adjusted for *MYCN* amplification, SCA and histology status (Table 1). When NTRK1 was excluded from the analysis, NTRK1-pY674/pY675 was significantly associated with enhanced 5-year RFS, strongly suggesting that it is an independent predictor of improved prognosis (Supplementary Table S3). Similar results were observed during analysis of wild-type *TP53* encoding tumors (Supplementary Tables S12 and S13) and non-amplified *MYCN* samples (Supplementary Tables S14 and S15). PTPN6 and TP53 overexpression was predictive of reduced RFS as shown for breast-cancer [19]. These findings suggest that components of this NGF-independent mechanism for NTRK1-Y674/Y675 phosphorylation [18] could be potential targets for neuroblastoma therapy.

Analysis of NTRK1-pY674/pY675 together with undetectable or low levels of PTPN6 and wild-type *TP53* expression in 52 tumors showed a significant association with improved RFS (*P*=0.01) (Table 3). The predictive value of NTRK1-pY674/pY675 together with low levels of PTPN6 followed the same trend as the result seen with the full data set. Furthermore, its presence was estimated, with the Kaplan–Meier method, to have a 48.48% probability of 5-year RFS with a median survival time of 4.9 years. This compared with only a 23.53% probability with a median survival time of 11.63 months when absent (*P*=0.042) (Supplementary Figure S1A).

Analysis of NTRK1-pY674/pY675 together with undetectable and low levels of PTPN6 and TP53 in non-amplified *MYCN* tumours revealed a significant association with improved 5-year RFS (*P*=0.001) (Table 4). In support of this result, the Kaplan–Meier method (Supplementary Figure S1B) estimated that tumors with this expression had 57.14% probability of 5-year RFS with a median survival time of over 5 years, whereas in its absence survival was reduced to a 31.58% probability with a median survival time of 3.49 years (*P*=0.062). Interestingly, univariate analysis of *MYCN* amplified samples for the presence of NTRK1-pY674/pY675 showed a trend towards prolonged 5-years RFS. Although, this result is most likely due to a low sample number, it supports the importance of NTRK1-pY674/pY675 expression for patient survival. Together these results suggest that in *MYCN* non-amplified tumors, the presence of NTRK1-pY674/pY675 together with undetectable or low levels of PTPN6 and TP53 is associated with improved 5-year RFS.

Tumors from patients younger than 18 months showed NTRK1-pY674/Y675 and undetectable or low levels of TP53 and PTPN6 (Table 6) significantly associated with improved 5-year RFS (78%) (*P*=0.007). Tumors of patients older than 18 months of age were associated with a trend towards improved RFS (50%) (*P*=0.135) (Supplementary Table S11). It was estimated, by Kaplan-Meier survival analysis, that tumors from patients younger than 18 months with Group 1 expression had 73.68% probability of 5-year RFS with a median survival time of over 5 years, whereas in its

absence, survival was reduced to 35% with a median survival time of 3.1 years (*P*=0.009) (Supplementary Figure S1C). Together these results are consistent for a role of NTRK1-pY674/pY675 and undetectable or low levels of PTPN6 and TP53 as a predictor of favorable outcome in patients younger than 18 months. Log-rank analysis of samples from patients older than 18 months of age gave a non-significant association which could be due to the low number of samples with Group 1 expression. Interestingly, an increase in chromosomal aberrations and the presence of *ATRX* gene mutations has been detected in tumors from patients older than 18 months of age but not from younger patients [29,30]. These findings suggest that other genetic changes could be contributing factors to this non-significant result.

Multivariate analysis of unfavorable/undifferentiating tumors for the presence of NTRK1-pY674/pY675 and low or undetectable levels of PTPN6 and TP53 showed significant association with 5-year RFS *(P*=0.007) (Table 5). Importantly, analysis of the different tumor groups adjusted for histology status demonstrates that lack of differentiation reveals a trend towards reduced relapse-free survival as seen by others [1,2]. The Kaplan–Meier method showed a 50% probability of 5-year RFS with a median survival time of 4.35 years in the presence of Group 1 expression whereas in its absence there was 15.38% probability of 5-year RFS with a median survival time of 6.64 months (*P*=0.008) (Supplementary Figure S1D).

Our results obtained with wild-type *TP53* encoding tumors support the importance of expression of NTRK1-pY674/pY675 and low or undetectable levels of PTPN6 and TP53 in neuroblastoma. To date the consensus in the TP53 field is that low TP53 protein expression is associated with wild-type *TP53* gene presence and high levels are associated with the presence of mutant *TP53* [10]. Although DNA from 60 neuroblastoma samples was sequenced, DNA of all 98 tumor samples in our cohort, needs to be sequenced in order to correlate the presence of wild-type or mutant *TP53* with PTPN6 and NTRK1-pY674/pY675 in the whole data for their predictive value. Our results in breast cancer show that expression of NTRK1-pY674/pY675 and low or undetectable levels of PTPN6 and TP53 are significantly associated with improved disease-free survival of patients [19].

We and others have demonstrated that PTPN6 protein overexpression is associated with grade III invasive breast tumours and is an indicator of reduced 15-year disease-free survival [19,31]. This investigation has revealed that PTPN6 overexpression is a possible independent predictor of poor 5-year RFS of neuroblastoma patients as multivariate analysis of unfavorable/undifferentiating tumors revealed PTPN6 to be significantly associated with reduced RFS with a hazard ratio of 4.03 (*P*=0.005) (Supplementary Table S8). Moreover, multivariate analysis of wild type TP53 expressing samples showed overexpression of PTPN6 significant associated with reduced RFS (hazard ratio of 3.87 (*P*=0.037) and 4.13 (*P*=0.001), respectively) (Supplementary Tables S12 and S13). This trend was observed across the analysis of the different tumor groups. Together these findings suggest that overexpression of PTPN6 is a poor prognostic predictor for neuroblastoma.

Our study is the first report describing a significant association between PTPN6 protein expression and reduced RFS of neuroblastoma patients.

Throughout this investigation it was possible to observe that presence of both *MYCN* amplification and SCA was associated with an increased risk of relapse. Our results demonstrate that in the absence of amplified *MYCN*, the presence of SCA are significantly associated with decreased RFS (hazard ratio= 3.02, *P*=0.045) (Supplementary Table S6). Similar results have been obtained by others [23].

Together these findings strongly suggest that the presence of NTRK1-pY675/pY675 together with undetectable or low TP53 and PTPN6 levels is an independent predictor of improved RFS of neuroblastoma patients. These observations support an important role for the mechanism of NTRK1-Y674/Y675 phosphorylation as a prognostic marker of neuroblastoma and are consistent with an NGF-independent NTRK1-Y674/Y675 phosphorylation via wild-type TP53 dependent repression of PTPN6 expression [18]. These results are in agreement with our previous investigation of 308 invasive breast tumors in which we demonstrated that the presence of NTRK1-pY675/pY675 together with undetectable or low TP53 and PTPN6 levels is significantly associated with 15-year disease-free survival of breast-cancer patients [18].

Moreover, these results suggest that components of this mechanism of NTRK1-Y674/Y675 phosphorylation could be targets for neuroblastoma therapy. NTRK1 provides such a potential therapeutic target [32], where a chemical compound that induces phosphorylation at tyrosines 674/675 could be investigated for possible treatment, similarly to CPPy and NS 1231 (which phosphorylate NTRK1 at Y490) shown to be possible candidates for neuroblastoma care [33,34]. Importantly, the stimulation of signalling pathways via NTRK1-pY674/pY675 involves the activation and binding of TID1, SH2B1 and c-abl which promote neurite outgrowth of PC12 cells and the maintenance of neonatal sympathetic neurons [16,35,36]. Given that cell differentiation is key to favorable prognosis [1,2], it will be important to identify the transcriptomic and proteomic profiles of tumors with this mechanism, in order to assess molecular commitment to differentiation in the absence of histological change. This question is currently being addressed by our group. Moreover, given the relevance of NTRK2 as a poor outcome prognostic marker, we will be assessing as to whether in the presence or absence of non-phosphorylated or phosphorylated NTRK2, NTRK1-Y674/Y675 and low or undetectable levels of TP53 and PTPN6 is associated with increased RFS of neuroblastoma patients. Despite significant research, neuroblastoma remains a childhood cancer associated with high patient mortality worldwide [1,2]. The presence of the NTRK1-pY674/pY675, PTPN6 and wild-type TP53 mechanism offers a potential candidate for prognosis and therapeutic intervention of neuroblastoma patients.

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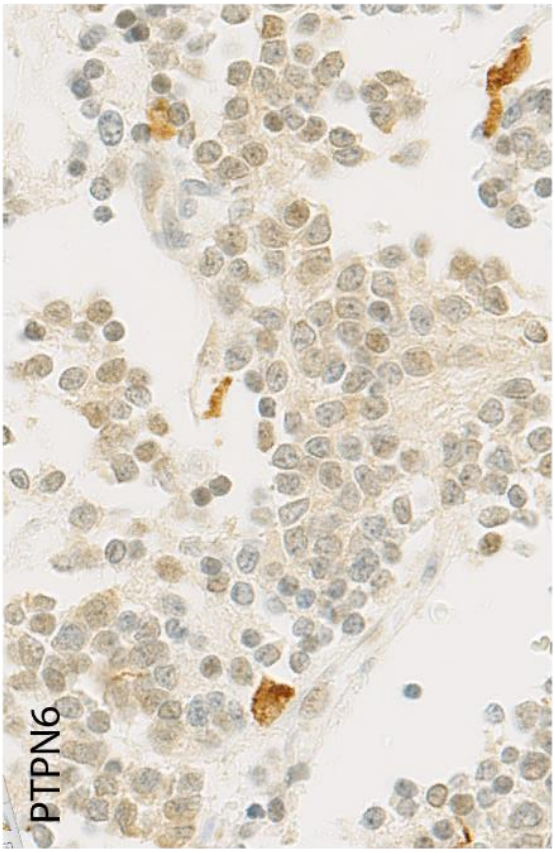
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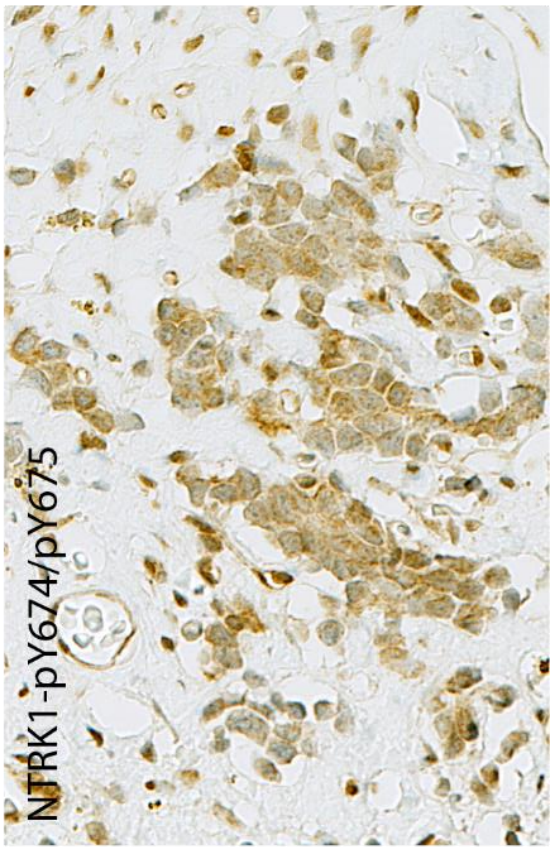
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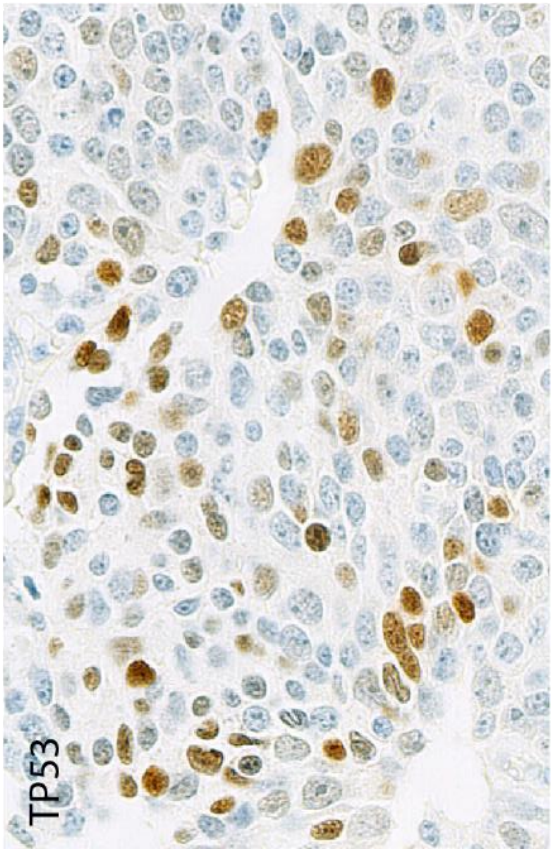
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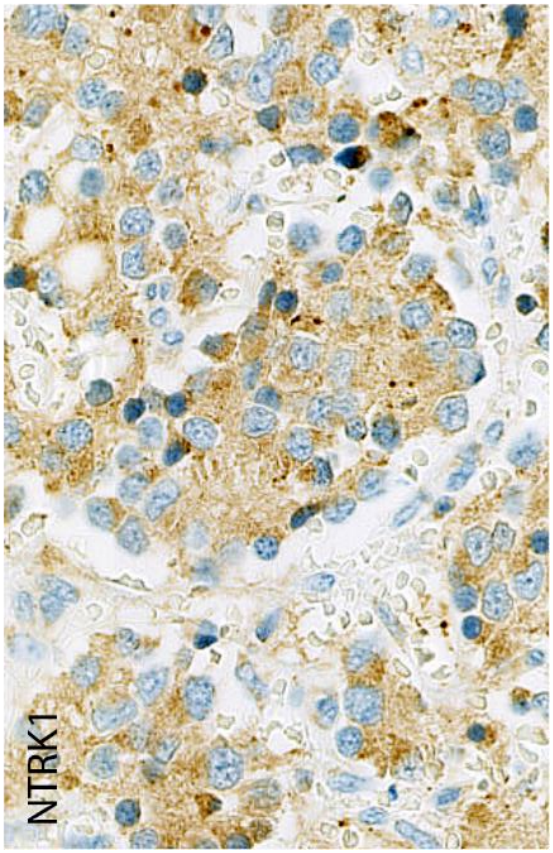
NTRK1

Figure 1. Immunohistochemical analysis of non-phosphorylated and phosphorylated NTRK1 at Y674/Y675, PTPN6, and TP53 in neuroblastoma tumour samples.

Immunostaining showed TP53 expression in the nucleus. PTPN6 was present in the nucleus and cytoplasm. NTRK1 was expressed as punctuate pattern, primarily, in the cytoplasm. Phosphorylated NTRK1 at Y674/Y675 was preferentially present in the cell membrane. Photography was carried out at X40 magnification.

Figure 2

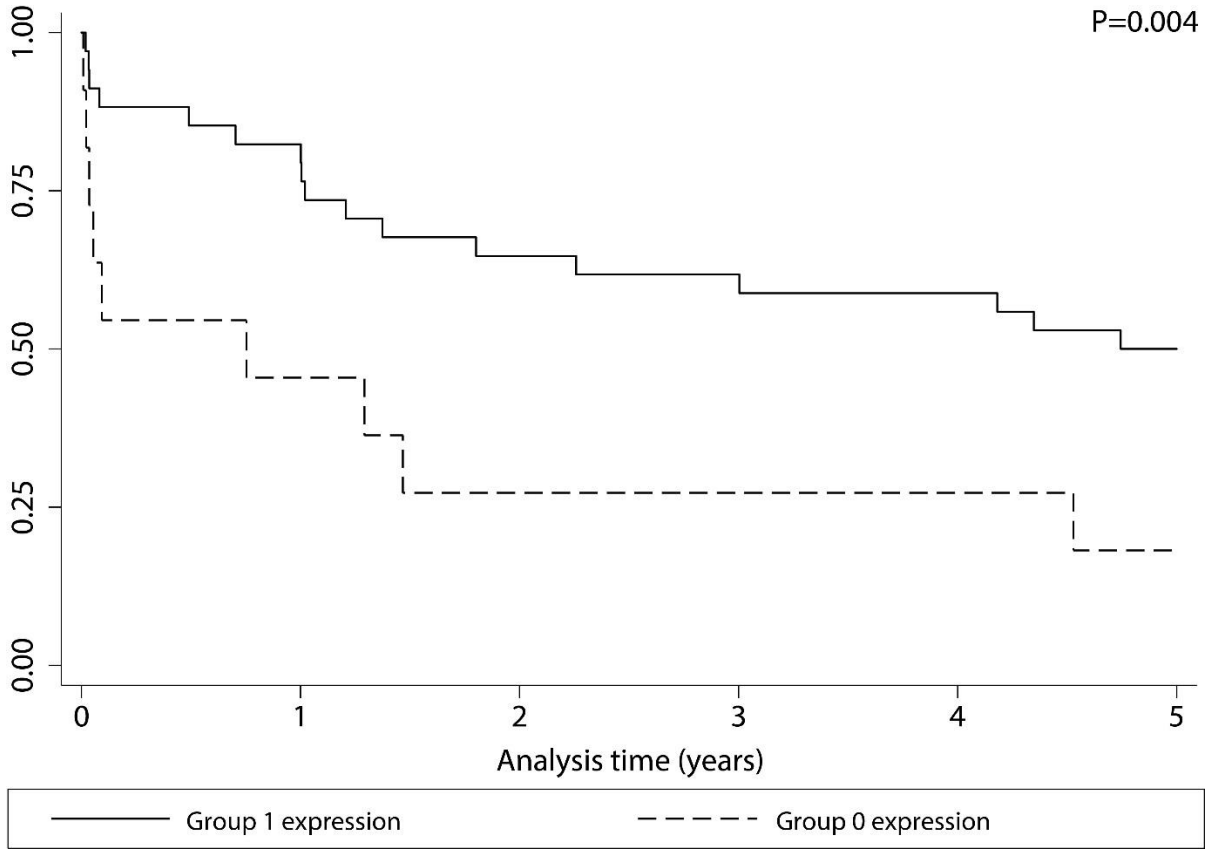
P=0.004
1.00
0.75
0.50
0.25
0.00
0
1
2
3
4
5
Analysis time (years)
Group 1 expression
Group 0 expression

Figure 2. Kaplan–Meir survival curve for 49 neuroblastoma tumors expressing phosphorylated NTRK1-Y674/Y675 together with undetectable or low levels of PTPN6 and TP53.

It was estimated that tumours with Group 1 expression (solid line) had 50% probability of 5-year relapse-free survival with a median survival time of 4.73 years. Whereas in its absence (dashed line), the possibility of survival was reduced to 19.51% with a median survival time of 11.63 months. The separation is significant as identified by the Log-rank test (P = 0.004).

Table 1. Multivariate analysis[a] of 98 neuroblastoma samples, expressing phosphorylated NTRK1-Y674/Y675, NTRK1, PTPN6 and TP53, for 5-year relapse-free survival.

Variable	5-year relapse-free survival	
	Hazard ratio [CI][b]	**P[c]**
NTRK1-pY674/pY675		
0	1	
1	0.56 [0.30-1.03]	0.063
2/3	0.40 [0.21-0.77]	**0.006**
NTRK1		
0	1	
1	0.62 [0.20-1.92]	0.408
2/3	0.26 [0.07-0.90]	**0.038**
PTPN6		
0	1	
1	0.93 [0.50-1.71]	0.808
2/3	1.69 [0.75-3.82]	0.204
TP53		
0	1	
1	0.90 [0.30-2.66]	0.844
2/3	1.55 [0.72-3.35]	0.265
***MYCN* amplification**		
Non-amplified	1	
Amplified	3.68 [1.60-8.47]	**0.002**
***SCA*[d]**		
Absence	1	
Presence	2.24[0.89-5.64]	0.088
Histological status[e]		
Favorable/differentiating	1	
Unfavorable/undifferentiating	1.49 [0.87-2.56]	0.147

[a]Multivariate analysis Cox Regression Model. **0:** Absence of expression **1:** Weak expression **2:** Moderate expression **3:** Strong expression

[b]CI confidence intervals.

[c]P value of statistical significance. *P* values lower than 0.05 were deemed significant.

[d]Segmental chromosomal abnormalities (SCA): 17q gain, 1p and 11q loss.

[e]Favorable/differentiating histological subtypes include: differentiating neuroblastoma, intermixed ganglioneuroblastoma and ganglioneuroma. Unfavorable/undifferentiating subtypes include: undifferentiating neuroblastoma and nodular ganglioneuroblastoma.

Table 2. Multivariate analysis[a] of 49 neuroblastoma samples, expressing phosphorylated NTRK1-Y674/Y675 together with undetectable or low levels of PTPN6 and TP53, for 5-year relapse-free survival.

Variable	5-year relapse-free survival	
	Hazard ratio[CI][b]	**P[c]**
Group 1 (NTRK1-pY674/pY675-PTPN6-TP53)[d]		
Absence	1	
Presence	0.36 [0.16-0.81]	**0.014**
***MYCN* amplification**		
Non-amplified	1	
Amplified	4.78 [2.09-10.9]	**<0.001**
SCA[e]		
Absence	1	
Presence	1.90 [0.79-4.52]	0.149
Histological status[f]		
Favorable/differentiating	1	
Unfavorable/undifferentiating	1.36 [0.78-2.36]	0.279

[a]Multivariate analysis Cox Regression Model. **0:** Absence of expression **1:** Weak expression **2:** Moderate expression **3:** Strong expression

[b]CI confidence intervals.

[c]P value of statistical significance. *P* values lower than 0.05 were deemed significant.

[d]Presence of phosphorylated NTRK1 at Y674/Y675 and undetectable or low expression of PTPN6 and TP53.

[e]Segmental chromosomal abnormalities (SCA): 17q gain, 1p and 11q loss.

[f]Favorable/differentiating histological subtypes include: differentiating neuroblastoma, intermixed ganglioneuroblastoma and ganglioneuroma. Unfavorable/undifferentiating subtypes include: undifferentiating neuroblastoma and nodular ganglioneuroblastoma.

Table 3. Multivariate analysis[a] of 52 wild-type TP53 expressing neuroblastoma samples, for the presence of NTRK1-pY674/pY675 together with undetectable or low levels of PTPN6, for 5-year relapse-free survival.

Variable	5-year relapse-free survival	
	Hazard ratio[CI][b]	**P[c]**
Group 1 (NTRK1-pY674/pY675-PTPN6-TP53)[d]		
Absence	1	
Presence	0.35 [0.16-0.78]	**0.010**
***MYCN* amplification**		
Non-amplified	1	
Amplified	3.50 [1.51-8.12]	**0.004**
SCA[e]		
Absence	1	
Presence	2.43 [0.89-6.58]	0.082
Histological status[f]		
Favorable/differentiating	1	
Unfavorable/undifferentiating	1.66 [0.67-4.08]	0.275

[a]Multivariate analysis Cox Regression Model. **0:** Absence of expression **1:** Weak expression **2:** Moderate expression **3:** Strong expression

[b]CI confidence intervals.

[c]P value of statistical significance. *P* values lower than 0.05 were deemed significant.

[d]Presence of phosphorylated NTRK1 at Y674/Y675, undetectable or low expression of PTPN6 and wild-type TP53.

[e]Segmental Chromosomal Abnormalities (SCA): 17q gain, 1p and 11q loss.

[f]Favorable/differentiating histological subtypes include: differentiating neuroblastoma, intermixed ganglioneuroblastoma and ganglioneuroma. Unfavorable/undifferentiating subtypes include: undifferentiating neuroblastoma and nodular ganglioneuroblastoma.

Table 4. Multivariate analysis[a] of 69 *MYCN* non-amplified neuroblastoma samples expressing phosphorylated NTRK1-Y674/Y675 together with undetectable or low levels of PTPN6 and TP53 for 5-year relapse-free survival.

Variable	5-year relapse-free survival	
	Hazard ratio[CI][b]	**P[c]**
Group 1 (NTRK1-pY674/pY675-PTPN6-TP53)[d]		
Absence	1	
Presence	0.33 [0.17-0.65]	**0.001**
SCA[e]		
No	1	
Yes	1.98 [0.68-5.79]	0.213
Histological status[f]		
Favorable/Differentiating	1	
Unfavorable/undifferentiating	1.07 [0.55-2.10]	0.841

[a]Multivariate analysis Cox Regression Model. **0:** Absence of expression **1:** Weak expression **2:** Moderate expression **3:** Strong expression

[b]CI confidence intervals.

[c]P value of statistical significance. *P* values lower than 0.05 were deemed significant.

[d]Presence of phosphorylated NTRK1 at Y674/Y675 and undetectable or low expression of PTPN6 and TP53.

[e]Segmental chromosomal abnormalities (SCA): 17p gain, 1q and 11q loss.

[f]Favorable/differentiating histological subtypes include: differentiating neuroblastoma, intermixed ganglioneuroblastoma and ganglioneuroma. Unfavorable/undifferentiating subtypes include: undifferentiating neuroblastoma and nodular ganglioneuroblastoma.

Table 5. Multivariate analysis[a] of 60 unfavorable/undifferentiating neuroblastoma samples expressing phosphorylated NTRK1-Y674/Y675 together with undetectable or low levels of PTPN6 and TP53 for 5-year relapse-free survival.

Variable	5-year relapse-free survival	
	Hazard ratio[CI][b]	**P[c]**
Group 1 (NTRK1-pY674/pY675-PTPN6-TP53)[d]		
Absence	1	
Presence	0.34 [0.16-0.75]	**0.007**
***MYCN* amplification**		
Non-amplified	1	
Amplified	4.43 [1.83-10.69]	**0.001**
SCA[e]		
Absence	1	
Presence	1.58 [0.61-4.12]	0.348

[a]Multivariate analysis Cox Regression Model. **0:** Absence of expression **1:** Weak expression **2:** Moderate expression **3:** Strong expression

[b]CI confidence intervals.

[c]P value of statistical significance. *P* values lower than 0.05 were deemed significant.

[d]Presence of phosphorylated NTRK1 at Y674/Y675 and undetectable or low expression of PTPN6 and TP53.

[e]Segmental chromosomal abnormalities (SCA): 17q gain, 1p and 11q loss.

[f] Unfavorable/undifferentiating subtypes include: undifferentiating neuroblastoma and nodular ganglioneuroblastoma.

Table 6. Multivariate analysis[a] of 41 neuroblastoma samples from patients younger than 18 months of age expressing phosphorylated NTRK1-Y674/Y675 together with undetectable or low levels of PTPN6 and TP53 for 5-year relapse-free survival.

Variable	5-year relapse-free survival	
	Hazard ratio[CI][b]	**P[c]**
Group 1 (NTRK1-pY674/pY675-PTPN6-TP53)[d]		
Absence	1	
Presence	0.22 [0.07-0.66]	**0.007**
***MYCN* amplification**		
Non-amplified	1	
Amplified	9.38 [1.75-50.24]	**0.009**
SCA[e]		
Absence	1	
Presence	1.77 [0.43-4.00]	0.421
Histological status[f]		
Favorable/differentiating	1	
Unfavorable/undifferentiating	1.32 [0.32-3.05]	0.628

[a]Multivariate analysis Cox Regression Model. **0:** Absence of expression **1:** Weak expression **2:** Moderate expression **3:** Strong expression

[b]CI confidence intervals.

[c]P value of statistical significance. *P* values lower than 0.05 were deemed significant.

[d]Presence of phosphorylated NTRK1 at Y674/Y675 and undetectable or low expression of PTPN6 and TP53.

[e]Segmental chromosomal abnormalities (SCA): 17q gain, 1p and 11q loss.

[f]Favorable/differentiating histological subtypes include: differentiating neuroblastoma, intermixed ganglioneuroblastoma and ganglioneuroma. Unfavorable/undifferentiating subtypes include: undifferentiating neuroblastoma and nodular ganglioneuroblastoma.