N-Myc regulates expression of the detoxifying enzyme glutathione transferase GSTP1, a marker of poor outcome in neuroblastoma

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Abstract

Amplification of the transcription factor MYCN is associated with poor outcome and a multidrug resistant phenotype in neuroblastoma. N-Myc regulates the expression of several ATP-binding cassette (ABC) transporter genes, thus affecting global drug efflux. Because these transporters do not confer resistance to several important cytotoxic agents used to treat neuroblastoma, we explored the prognostic significance and transcriptional regulation of the phase II detoxifying enzyme, glutathione S-transferase P1 (GSTP1). Using quantitative real-time PCR, GSTP1 gene expression was assessed in a retrospective cohort of 51 patients, and subsequently in a cohort of 207 prospectively accrued primary neuroblastomas. These data along with GSTP1 expression data from an independent microarray study of 251 neuroblastoma samples were correlated with established prognostic indicators and disease outcome. High levels of GSTP1 were associated with decreased event-free and overall survival in all three cohorts. Multivariable analyses, including age at diagnosis, tumor stage and MYCN amplification status, were conducted on the two larger cohorts, independently demonstrating the prognostic significance of GSTP1 expression levels in this setting. Mechanistic investigations revealed that GSTP1 is a direct transcriptional target of N-Myc in neuroblastoma cells. Together, our findings reveal that N-Myc regulates GSTP1 along with ABC transporters that act to control drug metabolism and efflux. Further, they imply that strategies to jointly alter these key multidrug resistance mechanisms may have therapeutic implications to manage neuroblastomas and other malignancies driven by amplified Myc family genes.
Introduction

Neuroblastoma is a solid tumor of embryonal neural crest origin and is the most common extracranial solid tumor of early childhood, accounting for 15% of all cancer related deaths in children (1). Patients diagnosed over one year of age often have disseminated, metastatic disease, often with MYCN amplification. While neuroblastomas typically respond to initial therapy regardless of risk group (2), the majority of patients with high-risk disease relapse with tumors that are refractory to treatment. There are no salvage regimens known to be curative for these patients (3). We have previously demonstrated that the ATP binding cassette transporter C1 (ABCC1), a key component of phase III detoxification, is a powerful independent prognostic marker in neuroblastoma (4, 5). Consistent with this observation, ABCC1 is able to efflux a wide range of chemotherapeutics (6), including many agents of importance in neuroblastoma therapy. However, several important chemotherapeutic agents to which resistance is observed, including cisplatin and cyclophosphamide, are not substrates of ABCC1. Amongst the well-characterized contributors to multidrug resistance in other types of tumors are cellular detoxification pathways. In particular, the Phase II conjugating enzyme glutathione S-transferase P1 (GSTP1) is up-regulated in a wide range of tumors and high GSTP1 expression is often associated with multidrug resistance (7-9). GSTP1 catalyses the conjugation of reduced glutathione to otherwise harmful electrophilic compounds, including cytotoxic drugs, and can sequester a range of non-substrate ligands (10). In addition to these roles, GSTP1 has been proposed to inhibit the mitogen-activated protein kinase (MAPK) pathway through direct interaction with c-Jun N-terminal kinase 1 (JNK1), decreasing sensitivity to drug-induced apoptosis (11). We have therefore extended our studies to investigate the prognostic value of this gene in neuroblastoma.

Here we demonstrate that GSTP1 expression is an independent prognostic indicator of clinical outcome in primary neuroblastoma and, like ABCC1, is a direct transcriptional target of the N-Myc oncoprotein. The regulation of multiple components of drug metabolism pathways has implications for understanding drug insensitivity in neuroblastoma patients with MYCN.
amplification, and potentially in other malignancies with increased expression of Myc family proteins.

Materials and Methods

Patient tumor samples

All patient material used for gene expression analyses has been previously described (5, 12). In brief, a small discovery cohort of 51 primary tumors from previously untreated patients was obtained from either the Sydney Children’s Hospital, Sydney, Australia, or the Neuroblastoma Tumor Bank of the Pediatric Oncology Group, USA (“SCH/POG” cohort) (12) and a large validation cohort of 207 patients enrolled onto COG Neuroblastoma Biology Study 9047 was obtained from the Tumor Bank of the Children’s Oncology Group, USA (“COG” cohort) (5). The study was approved by the individual institutional review boards, and informed consent was obtained for patients registered on the study. Publicly available data for GSTP1 expression from a customized oligonucleotide microarray dataset of primary tumors from 251 neuroblastoma patients from the German Neuroblastoma Trials NB90-NB2004 were also analysed as a second independent validation cohort (“Oberthuer cohort”; GSTP1 probe = A_23_P202658). The patient cohort and microarray study are previously published (13). A summary of patient data for each cohort is provided as Supplementary data Tables 1, 2 and 3.

Treatment

Treatments subsequently administered to patients were specific to their disease stage, age and tumor biology and have been previously described for each of the SCH/POG (4), COG (5) and Oberthuer cohorts (14).

TH-MYCN mouse tumor samples
The TH-MYCN transgenic mouse model of neuroblastoma, which has the expression of the human MYCN oncogene targeted to mouse neuroectodermal cells via the rat tyrosine hydroxylase promoter, has been previously described (15-17). When medium palpable tumors (approximately 1 cm²) were detected, they were resected, measured, and snap-frozen in liquid nitrogen for subsequent analysis. All experimental procedures were approved by the University of New South Wales Animal Care and Ethics Committee and were conducted under the Animal Research Act, 1985 (New South Wales, Australia) and the Australian Code of Practice for the Care and Use Animals for Scientific Purposes (1997).

**RT-PCR methods**

For tumor samples, total cytoplasmic RNA was isolated from human and mouse tumor material using a guanidinium thiocyanate-phenol-chloroform extraction method (4) and cDNAs were synthesized from 1 μg of RNA using random hexanucleotide primers and Moloney murine leukemia virus reverse transcriptase. Gene expression was determined by real-time polymerase chain reaction (RT-PCR) using either a Prism 7700 or 7900 Sequence Detection System (Applied Biosystems, Foster City, CA) with the following oligonucleotide primers and probes: human GSTP1, beta2-microglobulin (B2M) and MYCN, and mouse Gstp1, Gstp2 (the two mouse genes corresponding to human GSTP1) and B2m. All sequences are shown in Supplementary data Table 4.

For cell lines, RNA samples were prepared using Tri-Reagent (Sigma-Aldrich, St Louis, MO) and treated with DNase (DNAfree; Ambion, Austin, TX). Reverse transcription and PCR were performed using a SuperScript reverse transcription-PCR kit (Invitrogen, Carlsbad, CA). RT-PCR was performed using iQ SYBR Green Supermix and the iQCycler thermocycler (Bio-Rad, Hercules, CA) with primers and probes for human MYCN, GSTP1, NUC and GUSB. All sequences are shown in Supplementary data Table 4.

Gene expression levels were determined using the ΔΔCt method, normalized to the B2M control, and expressed relative to a calibrator (18).
Western blot analysis

Frozen tissue (approximately 50 mg) was pulverized on dry ice with a mortar and pestle in 500 μL lysis buffer (2 x PBS, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) containing a protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO). Samples were centrifuged at 13,000 RPM in a benchtop centrifuge and the supernatants retained and quantitated by BCA assay (Pierce, Rockford, IL). Samples (20 μg) were electrophoresed on a 4-20% SDS-PAGE gel and transferred to nitrocellulose membrane (GE Healthcare, Rydalmere, NSW, Australia). The membrane was blocked with 5% skim milk powder in Tris buffered saline with 0.05% Tween 20, then incubated with an anti-GSTP1 antibody (mouse monoclonal 3F2C2, Abcam, Cambridge, UK) followed by a horseradish peroxidase conjugated secondary antibody. Membranes were developed using Supersignal reagent (Pierce, Rockford, IL), visualized on film and quantitated relative to α-tubulin by densitometry using QuantityOne software (BioRad, Hercules, CA).

Human neuroblastoma cell lines and culture

SH-EP Tet-21N cells (19) were cultured in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated tetracycline-free fetal bovine serum, while BE(2)-C and LA-N-5 cells were cultured in RPMI medium 1640 containing 20% fetal bovine serum and 50 mg/mL gentamycin. For MYCN repression, SH-EP Tet-21N cells were treated with 2μg/mL tetracycline for the indicated time. Cell lines were obtained from: Manfred Schwab (SH-EP Tet-21N), German Collection of Microorganisms and Cell Cultures (BE(2)-C) and Deborah Tweddle (LA-N-5) between 1997 and 2005, and were systematically validated for MYCN copy number and expression of specific markers such as MYCN, upon receipt and during usage by dr. Giuliano Della Valle who runs the cytogenetic facility, Department of Biology, University of Bologna, Italy.

Chromatin immunoprecipitation (ChIP) assays
The promoter region of *GSTP1* was analysed using bioinformatics techniques to identify putative N-Myc binding sites, including the canonical E-box sequence CACGTG and other non-canonical sequences such as CATGTG and CACGCG. Promoter regions from -2000 to +2000 base pairs with respect to the transcription start site were considered for analysis. To test binding of N-Myc, quantitative ChIP was performed as previously described (20, 21) using either SH-EP Tet-21N cells cultured in the absence of tetracycline (presence of N-Myc), BE(2)-C or LA-N-5 cells. Antibodies employed in this study were: IgG (Santa Cruz Biotechnology, Santa Cruz, CA, sc-2027); N-Myc monoclonal antibody B8.4.B (22) and Max C-17 (Santa Cruz Biotechnology, sc-197). Specific pairs of primers used for quantitative ChIP are listed in Supplementary data Table 5.

**Statistical analysis**

The relationship between levels of target gene expression and patient survival was analyzed using the Kaplan-Meier method with curves analyzed by log-rank test. Standard errors were calculated according to the method of Peto and Peto (23). Comparisons of outcome between subgroups in multivariable analyses were performed using Cox’s proportional hazards regression model. The relationship between levels of target gene expression was analysed using Spearman’s rank correlation. Differences in target gene expression between samples were assessed using a two-sided Student’s *t* test. Statistical analyses were conducted using either Stata (StataCorp, College Station, TX) or SPSS (IBM, Chicago, IL). Event Free Survival (EFS) time was calculated from the time of enrolment onto the relevant study, or from diagnosis (SCH samples), to the time of the first occurrence of an event (relapse, progressive disease, secondary malignancy, or death) or to the date of last contact if no event occurred. Death was the only event considered for the calculation of overall survival time. All samples were de-identified, and researchers who performed the gene expression analysis were blinded to all clinical characteristics and outcome of the subjects.

Tumors were categorized as having high expression of *GSTP1* based on a range of cut points, including the median, upper quartile, and upper decile PCR values. The statistical
methodology used to identify the optimal cut point to maximize the difference in outcome between groups with low versus high expression has previously been described in detail (24).

Results

Prognostic significance of GSTP1 in primary human neuroblastoma

In order to assess the prognostic significance of GSTP1 expression in neuroblastoma, we initially used RT-PCR to analyse its expression in a small cohort of 51 primary untreated tumors. When patients were dichotomized around the median real-time PCR value, high expression of GSTP1 was significantly associated with poorer event free survival ($P = 0.002$, Figure 1A) with 5-year survival rates of 92% ± 5.4% and 51% ± 11% for tumors with low and high GSTP1 expression, respectively, and also significantly associated with poorer overall survival ($P = 0.006$, Figure 1B), with 5-year survival rates of 96% ± 3.9% and 63% ± 10% for tumors with low and high GSTP1 expression, respectively. Furthermore, GSTP1 expression was significantly higher in tumors with MYCN amplification than in non-amplified tumors (Figure 1C, $P = 0.024$). To verify that GSTP1 gene expression accurately reflected expression at the protein level, we conducted Western blotting on six representative tumor samples, three with high GSTP1 gene expression and three with low gene expression (Figure 1D). Protein levels were found to correlate strongly with gene expression in these samples (Figure 1E; $r = 0.96$, $P = 0.002$). Full-length Western blots are presented in Supplementary Figure S1.

Next, we extended these observations to two large patient cohorts. In the first, we analysed GSTP1 expression by RT-PCR in 207 primary tumors (COG cohort). In the second, we analysed publicly available microarray data (13) for GSTP1 expression in 251 primary tumors (Oberthuer cohort).

In the COG cohort, high GSTP1 expression was associated with worse outcome when patients were dichotomized around either the median or upper quartile real-time PCR values, although this failed to reach statistical significance at these cut-points (not shown). However at the
upper decile, high GSTP1 expression was significantly associated with reduced EFS ($P = 0.002$; Figure 2A). The 5-year EFS rate for patients with high GSTP1 expression was $52\% \pm 11\%$, compared with $75\% \pm 3.3\%$ for low GSTP1 expression. The prognostic significance of GSTP1 was also examined for patients with tumors lacking MYCN amplification. While these patients would be expected to have a relatively favourable outcome compared to patients with MYCN amplification, high levels of GSTP1 expression remained predictive of poorer outcome with a 5-year EFS rate of $60\% \pm 13\%$ compared to $80\% \pm 3\%$ for patients with low GSTP1 expression in their tumors ($P = 0.025$; Figure 2B). Similarly, within poor prognosis patients (INSS stage 3 and 4), high levels of GSTP1 expression identified a subset with particularly poor outcome and a 5-year EFS rate of only $31\% \pm 14\%$ compared to $52\% \pm 6\%$ for patients compared to patients with low GSTP1 expression ($P = 0.022$; Figure 2C). In each case, similar effects were observed for overall survival (not shown).

Within the Oberthuer cohort, GSTP1 was strongly associated with EFS at the upper decile ($P < 0.001$; Figure 2D), and also at the upper quartile and median (not shown). At the upper decile, the 5-year EFS rates for patients with high GSTP1 expression were $24\% \pm 13\%$ compared with $74\% \pm 3\%$ for low GSTP1 expression. In patients with tumors lacking MYCN amplification, 5-year EFS rates were $26\% \pm 20\%$ and $78\% \pm 3\%$ for those with high and low GSTP1 expression respectively ($P < 0.001$, Figure 2E). For those with poor prognosis (stage 3,4), high GSTP1 expression was associated with significantly worse outcome with 5-year EFS rates of $15\% \pm 12\%$ and $55\% \pm 6\%$ for high and low GSTP1 expression respectively ($P = 0.007$; Figure 2F). Within the stage 4 patients only, EFS was not significantly different for those with low and high GSTP1 expression in their tumors ($P = 0.227$), however overall survival rates retained significance ($P = 0.049$) with 5 year survival rates of $51\% \pm 8.5\%$ and $37\% \pm 14\%$ for high and low GSTP1 expression respectively (Supplementary Figure S2).

Multivariable analysis was performed to determine whether GSTP1 expression had prognostic significance independent of established prognostic indicators for neuroblastoma. Tumor stage (1, 2, or 4S vs 3 or 4), age at diagnosis (<12 mo vs $\geq$12 mo), MYCN status (amplified vs single
copy) and dichotomized GSTP1 expression were tested in a Cox’s proportional hazards model (Table 1). In the COG cohort, GSTP1 expression retained independent prognostic significance for EFS (hazard ratio = 2.6; 95% CI 1.2 to 5.5; \( P = 0.011 \)) and overall survival (hazard ratio = 2.21; 95% CI 1.0 to 4.8; \( P = 0.047 \)). Likewise in the Oberthuer cohort, GSTP1 expression retained prognostic significance for both EFS (relative risk = 2.0; 95% CI 1.1 to 3.8; \( P = 0.029 \)) and overall survival (relative risk = 2.3; 95% CI 1.1 to 4.9; \( P = 0.024 \)). Finally, in a multivariable analysis for stage 4 patients only, including GSTP1 expression and age, GSTP1 expression approached significance for overall survival (relative risk = 2.1; 95% CI 0.99 to 4.6; \( P = 0.054 \)), but was not significant for event-free survival (Supplementary data Table 6).

**Expression of GSTP1 correlates with MYCN in neuroblastoma tumors**

As amplification of the transcription factor MYCN is a common feature of poor outcome in neuroblastoma, and GSTP1 expression was higher in MYCN-amplified tumors than in those without MYCN amplification (Figure 1C), we sought to determine whether GSTP1 expression was regulated by MYCN. First, we assessed the correlation between GSTP1 expression and MYCN expression in the COG cohort after conducting RT-PCR analysis to determine relative MYCN expression levels. Using Spearman’s rank correlation, GSTP1 levels were found to correlate with MYCN expression in both tumors with MYCN-amplification (\( r = 0.61, P = 0.003 \); Figure 3A) and those without (\( r = 0.73, P < 0.001 \); Figure 3B). We also extended this analysis to the TH-MYCN transgenic mouse model of neuroblastoma (15), where the human MYCN transgene is expressed in cells of neuroectodermal origin by use of a tyrosine hydroxylase promoter. Using RT-PCR analysis, MYCN levels were found to correlate particularly closely with the expression of mouse Gstp1 (\( r = 0.91, P < 0.001 \); Figure 3C). Similar results were observed for Gstp2 (not shown). Finally, we assessed the expression of GSTP1 using RT-PCR at various time points after tetracycline-induced suppression of exogenous MYCN expression in the neuroblastoma cell line SH-EP Tet-21N (19). GSTP1 expression was decreased after tetracycline exposure, along with MYCN (not shown).
Collectively, these data support regulation of GSTP1 expression by N-Myc in cultured neuroblastoma cell lines, human neuroblastoma tumors, and tumors from a biologically relevant mouse model of neuroblastoma.

N-Myc directly regulates GSTP1 expression

In light of these results, we assessed whether N-Myc directly regulates GSTP1 expression. The promoter region of GSTP1 was examined bioinformatically for putative N-Myc-binding sites within 2000bp on either side of the transcriptional start site. Two canonical (CACGTG) and four non-canonical (CACGCG, CATGTG) E-box sites were identified over five distinct regions of the promoter (Figure 4A). Next, we used ChIP assays to examine the physical association of N-Myc with each of the identified regions using the three independent human neuroblastoma cell lines SH-EP Tet-21N, LA-N-5 and BE(2)-C. In each cell line, the GSTP1 promoter was directly bound by N-Myc, as well as its dimerization partner Max, at a non-canonical E-box proximal to the putative transcriptional start site (Fig. 4A). The fold enrichment for GSTP1 was comparable to that of APEXI, a well-known N-Myc target gene (25) indicating that the N-Myc/Max complex is strongly bound to the promoter.

Finally, to determine whether N-Myc directly activates GSTP1 transcription, we cloned the GSTP1 promoter upstream of a luciferase reporter gene and tested activity in SH-EP Tet-21N cells as a function of N-Myc expression. Luciferase activity was found to be dependent on N-Myc expression for the GSTP1 reporter construct, as for ABCC1, a positive control (26) (Figure 4B). Together, these data demonstrate that GSTP1 is a direct transcriptional target of N-Myc in neuroblastoma cells.

Discussion

Using three independent patient cohorts, we have shown that high expression of GSTP1 is a marker of poor prognosis in primary neuroblastoma. Furthermore, when included in multivariable...
analyses with the established prognostic indicators of age at diagnosis, MYCN amplification status and tumor stage as variables, GSTP1 expression retained independent prognostic significance. This result raises the possibility that GSTP1 contributes to the clinical behaviour of neuroblastoma, consistent with its known roles in drug resistance and tumor biology. While neuroblastomas typically respond to initial therapy regardless of risk group (2), the majority of patients with high-risk disease relapse with tumors that are refractory to treatment. It is as yet unclear whether this reflects the acquisition of multidrug resistance following exposure to chemotherapy, as a result of induction of one or more of the mechanisms which have previously been implicated as mediating multidrug resistance in neuroblastoma, such as MRP1 over-expression (5) or p53 mutation (27), or the selection and expansion of pre-existent multidrug resistant tumor clones, as we have previously described for acute lymphoblastic leukaemia (28). Future studies involving matched pairs of pre- and post-treatment tumor samples would be valuable to determine whether levels of GSTP1 or other drug-resistance associated genes are elevated at relapse compared to levels at the time of diagnosis.

We also provide evidence that GSTP1 is a direct transcriptional target of N-Myc. Firstly, expression of GSTP1 correlates with that of MYCN in both human tumors and the tumors derived from the TH-MYCN mouse neuroblastoma model. Secondly, the N-Myc protein, in complex with its dimerization partner Max, directly interacts with a non-canonical E-box proximal to the putative transcriptional start site of GSTP1. Finally, repression of MYCN expression reduces activity in a luminescent reporter assay using the GSTP1 promoter. Notably, both the multidrug transporter ABCC1, a Phase III detoxification component that can efflux a number of glutathione conjugated chemotherapeutic agents (29) and ABCC4, which can efflux both irinotecan and topotecan (12), are also independently prognostic of neuroblastoma outcome (4, 5, 12, 30) as well as directly regulated by N-Myc (26). MYCN amplification can therefore contribute to multidrug resistance in neuroblastoma through the upregulation of a suite of drug resistance genes covering a range of chemotherapeutic drugs. While previous in vitro studies suggest co-operativity between ABCC1 and GSTs (31, 32), GSTP1 has a relatively weak affinity for many anti-cancer drugs (7), implying
that these proteins do not necessarily form sequential steps for the detoxification of a particular drug.

It is notable that of the two cohorts analyzed by RT-PCR, \textit{GSTP1} expression was significantly associated with poor outcome when dichotomized around the median RT-PCR value in the initial SCH/POG 51 sample cohort, while in the larger COG cohort, the association between high \textit{GSTP1} expression and poor outcome was evident but not significant at this cut-point and in fact did not achieve prognostic significance until the data was dichotomized around the upper decile RT-PCR value. One contributing factor for this difference is most likely due to the differences between the patient characteristics of the two cohorts. Specifically, in the retrospectively accrued SCH/POG cohort of 51 tumors, 23.5\% of tumors were \textit{MYCN}-amplified and 55\% were INSS stage 3 or 4 (12), whilst in the prospectively accrued COG cohort (5), only 10.1\% of tumors were \textit{MYCN}-amplified and 42\% were INSS stage 3 or 4. The under-representation of \textit{MYCN} amplified, advanced stage tumors in the COG cohort can be related to the frequent diagnosis of stage 4 disease based on bone marrow sampling rather than tumor biopsy in that cohort, as we have previously described (5). As \textit{GSTP1} is transcriptionally regulated by \textit{MYCN}, and given the strong correlation between \textit{MYCN} amplification and \textit{GSTP1} overexpression, it is therefore not surprising that \textit{GSTP1} expression is more predictive of outcome in a cohort with a higher proportion of \textit{MYCN}-amplified tumors and less predictive in a cohort where \textit{MYCN}-amplified tumors are under-represented. Indeed, those \textit{MYCN} amplified patients lost to the COG cohort would most likely only further strengthen the association between high \textit{GSTP1} expression and poor patient outcome evident in that cohort. The distributions of RT-PCR values for each cohort are shown in Supplementary Figure S3.

The identification of \textit{GSTP1} as an N-Myc target gene also has importance for malignancies with high expression or amplification of c-Myc. N-Myc and c-Myc share a common set of target genes and are functionally interchangeable in the mouse (33). Furthermore, \textit{GSTP1} was one of a large number of genes identified as potential c-Myc transcriptional targets in a high-throughput ChIP study (25) and \textit{GSTP1} is transcriptionally regulated by c-Myc in osteosarcoma cell lines (GP,
manuscript in preparation). Finally, as G 

Gstpl is tightly coupled to MYCN expression in neuroblastomas arising in TH-MYCN mice, this is likely to be a suitable pre-clinical model in which to assess the efficacy of GSTP1 inhibitors.

While the most familiar role for GSTP1 is the conjugation of reduced glutathione to cytotoxic drugs, GSTP1 may possess other functions that contribute to chemoresistance, including inhibition of JNK1 (34), and activation of extracellular signal regulated kinase (ERK) (35). In addition to potential roles in mediating drug resistance in neuroblastoma, GSTP1 could potentially impact other tumor properties through its ability to conjugate the cyclopentenone prostaglandins 15d-Δ12,14-PGJ2 and PGA2 (36), leading to their export through ABCC1 and ABCC3 (37), and its ability to sequester 15d-Δ12,14-PGJ2 in the cytosol independently of conjugation (38). Both mechanisms may prevent cyclopentenone prostaglandins from binding to the nuclear localized gamma isoform of the peroxisome proliferator-activated receptor (PPAR-γ), activation of which is anti-proliferative, pro-differentiation and pro-apoptotic (39) in cancer cells. In neuroblastoma, activation of PPAR-γ by 15d-Δ12,14-PGJ2 has been shown to induce apoptosis (40) and differentiation (41). While we cannot exclude these additional functions in our experiments, analysis of microarray data available for the Oberthuer cohort did not reveal any evidence of an association between GSTP1 expression and that of JNK1 (MAPK8), ERK1 (MAPK1) or PPARγ (PPARG; Supplementary Figure S4), or with pathways associated with these genes (data not shown).

GSTP1 is up-regulated in numerous human tumors and high GSTP1 expression is associated with chemoresistance (7-9). To our knowledge, this is the first study to reveal the prognostic significance of GSTP1 expression in neuroblastoma and the first to demonstrate that GSTP1 is a direct transcriptional target of the MYCN oncogene, which is frequently amplified in poor-outcome neuroblastoma. These observations have significance both for MYCN-amplified neuroblastoma and for other diseases with amplification of Myc family oncogenes.
References

Table 1

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Variables adjusted for in the multivariable analysis included tumor stage (1, 2, 4S vs 3, 4), age at diagnosis (<12 months vs ≥ 12 months), MYCN status (amplified vs non-amplified), and GSTP1 expression (dichotomized at the upper decile low vs high).
Figure and table legends

Table 1: Multivariable Cox regression analysis of prognostic factors for neuroblastoma outcome.

Figure 1: GSTP1 is marker of poor-prognosis in neuroblastoma.
Kaplan-Meier curves for event free survival (A) and overall survival (B) in 51 NB patients from the SCH/COG cohort according to the expression of GSTP1. The level of GSTP1 expression was determined by RT-PCR and tumors dichotomized around the median value. Survival curves were analysed by log-rank test. (C) GSTP1 is expressed at a higher level in MYCN-amplified tumors by comparison with those without MYCN amplification. Graph shows relative expression as determined by ΔΔCT method ± SD (see Materials and Methods) with P value determined by Student’s t-test. (D) GSTP1 protein levels in six representative neuroblastoma tumors. Relative expression at mRNA level is shown above the blots, while protein expression relative to α-tubulin, as determined using densitometry, is shown below. The drug resistant neuroblastoma cell line BE(2)-C is used as a positive control. (E) GSTP1 mRNA levels are closely correlated with GSTP1 protein levels in primary neuroblastoma tumors (r = 0.96, P = 0.002). The relationship was analysed using linear regression.

Figure 2: GSTP1 is prognostic for outcome in two large, independent neuroblastoma cohorts.
Kaplan-Meier curves for neuroblastoma patients from the COG and Oberthuer cohorts showing EFS for the overall cohorts (A, D), patients with tumors lacking MYCN amplification (B, E), and those from patients of INSS stage 3 or 4 (C, F), according to the expression of GSTP1. In each case, tumors were dichotomized into “low” and “high” expression around the upper decile value. Survival curves were analysed by log-rank test. Five year EFS rates are indicated.

Figure 3: GSTP1 gene expression correlates with that of MYCN.
Expression of \( GSTP1 \) correlates with that of \( MYCN \) in tumors from the COG cohort either with MYCN-amplification (A), or without (B), while \( Gstp1 \) expression correlates with that of the \( MYCN \) transgene in tumors from the TH-\( MYCN \) mouse neuroblastoma model (C). The relationships were analysed using Spearman’s rank correlation.

**Figure 4:** \( GSTP1 \) is a direct transcriptional target of N-Myc.

(A) N-Myc associates with the \( GSTP1 \) promoter. Quantitative ChIP was applied to the neuroblastoma cell lines BE(2)-C, LA-N-5, and SH-EP Tet21/N cells expressing \( MYCN \). Relative enrichment is compared to the pre-immune serum. The results represent the mean ±S.E. of five independent ChIP experiments in which each region was amplified by RT-PCR in triplicate. Bent arrow, transcription start site; gray arrow, canonical E-box; black arrow, noncanonical E-box; black boxes, amplicons. The chromosome and coordinates (bp) are also given. Quantitative ChIP for \( APEX1 \) in BE(2)-C cells is also shown. (B) N-Myc regulates \( GSTP1 \) in a luciferase reporter assay. Firefly luciferase activity was determined following transient transfection of either a \( GSTP1 \) or \( ABCC1 \) reporter construct into SH-EP Tet21/N cells. Cells were either not treated (NT; MYCN ON), or treated with tetracycline for 24 or 48 h (TET 24h, or TET 48h; MYCN OFF). Cloned regions are shown at left, with bent arrows denoting the transcription start site, the cloned DNA region (bp) indicated below the promoter map and fragment coordinates expressed relative to the transcription start point. The results are the means of the relative fluorescence units (RFU) ±S.E. of three independent transfections.
Figure 1: GSTP1 is a marker of poor prognosis in neuroblastoma

A

B

C

D

E

GSTP1 is a marker of poor prognosis in neuroblastoma.
Figure 2: GSTP1 is prognostic for outcome in two, large independent neuroblastoma cohorts

A. COG cohort
   - All patients
     - Low (n=186): 75% EFS, P = 0.002
     - High (n=21): 52% EFS
   - Analysis time (d)

B. No MYCN amplification
   - Low (n=169): 80% EFS
   - High (n=15): 60% EFS, P = 0.025
   - Analysis time (d)

C. Stage 3 and 4 patients
   - Low (n=74): 52% EFS
   - High (n=12): 31% EFS, P = 0.022
   - Analysis time (d)

D. Oberthuer cohort
   - All patients
     - Low (n=227): 74% EFS
     - High (n=24): 24% EFS, P < 0.001
   - Analysis time (d)

E. No MYCN amplification
   - Low (n=202): 78% EFS
   - High (n=15): 26% EFS, P < 0.001
   - Analysis time (d)

F. Stage 3 and 4 patients
   - Low (n=87): 55% EFS
   - High (n=18): 15% EFS, P = 0.007
   - Analysis time (d)
Figure 3: GSTP1 expression correlates strongly with that of MYCN in tumours.

A
Tumors with MYCN amplification

B
Tumors without MYCN amplification

C
TH-MYCN mouse tumors
Figure 4: GSTP1 is a direct transcriptional target of N-Myc
N-Myc regulates expression of the detoxifying enzyme glutathione transferase GSTP1, a marker of poor outcome in neuroblastoma

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