Recurrent *NMYC* copy number gain and high protein expression in basal cell carcinoma

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Abstract. Formation of basal cell carcinoma (BCC) has been linked to deregulation in the sonic hedgehogh (Shh) signalling pathway. Though mutations of the genes, *PTCH1* and *SMO,* are known to be involved in aberrant Shh signalling, the distinct downstream effectors of these genes are poorly described. Studies have indicated that the *NMYC* oncogene is a potential Shh downstream effector. To assess the expression of Nmyc protein and gene copy numbers of the *NMYC* gene locus in a representative BCC tumour collection, immunohistochemistry (IHC) and fluorescence *in situ* hybridisation (FISH) were performed on 273 BCC specimens of different growth patterns and anatomic localisations on tissue microarray (TMA) sections. High Nmyc protein expression was detected in 72.7% (160/220) of all BCC specimens. Strong Nmyc immunopositivity was more frequently found in infiltrative BCCs compared to nodular/superficial BCCs (p=0.005), and in BCCs of the head compared to BCCs of other anatomic localisations (p=0.021). The prevalence of *NMYC* copy number gains was 17.5% (37/211), including three tumours with nodular differentiation that exhibited a distinct high-level amplification of the *NMYC* locus. These data indicate that high expression of the Shh downstream mediator, Nmyc, is a frequent event in BCC, predominantly in more aggressive subtypes. Although the *NMYC* copy number gain found in a subset of cases might contribute to this aberrant Nmyc protein expression by a gene dosage effect, our data suggests that Nmyc protein can also be induced by aberrant Shh signalling, acting as an effector molecule of the Shh pathway. Novel systemic anti-sense *NMYC* inhibition strategies could be a promising option for therapy-refractory BCC.

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Introduction

Basal cell carcinoma (BCC) of the skin is the most frequent neoplasm in humans, accounting for approximately 75% of all skin cancers (1). Mortality rates are generally low since BCC has a limited capacity for the formation of metastases (2). However, due to an infiltrative growth pattern, extensive tissue destruction can result if treatment failure occurs (3).

Histopathological classification of BCC is usually performed according to the predominant growth pattern. The most common subtype is the nodular type, accounting for approximately 50-60% of all BCCs, which is characterised by rounded masses of tumour cells within the dermis exhibiting prominent peripheral palisading. Microcystic and adenoid structures can also appear within nodular BCC. The superficial subtype is composed of small buds of proliferating cells growing down from the epidermis into the superficial dermis frequently accompanied by a diffuse inflammatory cell infiltrate. These two subtypes are considered to inherit a less aggressive clinical course with lower rates of relapsed tumours than the infiltrative (morphoeic) subtype, which usually consists of nests of irregular tumour cells of varying size embedded in a dense sclerotic fibrous stroma of collagen fibres (4).

BCC pathogenesis has been strongly linked to aberrations in the *hedgehog* signalling pathway. The tumour-suppressor gene, *PTCH1,* encodes a transmembrane protein that together with the *SMO* gene product (Smoh/smoothened) forms the receptor complex for the sonic hedgehog (Shh) signalling molecule. In Gorlin's syndrome, a rare hereditary disorder featuring the development of multiple BCCs at an early age, inactivating germline mutation of *PTCH1* was described together with somatic alteration of the second allele in the corresponding tumour (5). Biallelic *PTCH1* alterations were also found in sporadic BCC (6). Furthermore, *SMO* activating somatic missense mutations were reported, which caused constitutive, ligand-independent signal transduction resulting in neoplastic growth (7). The assumed downstream effectors of deregulated hedgehog signalling are Gli1, Gli2, Pdgfrα (8)

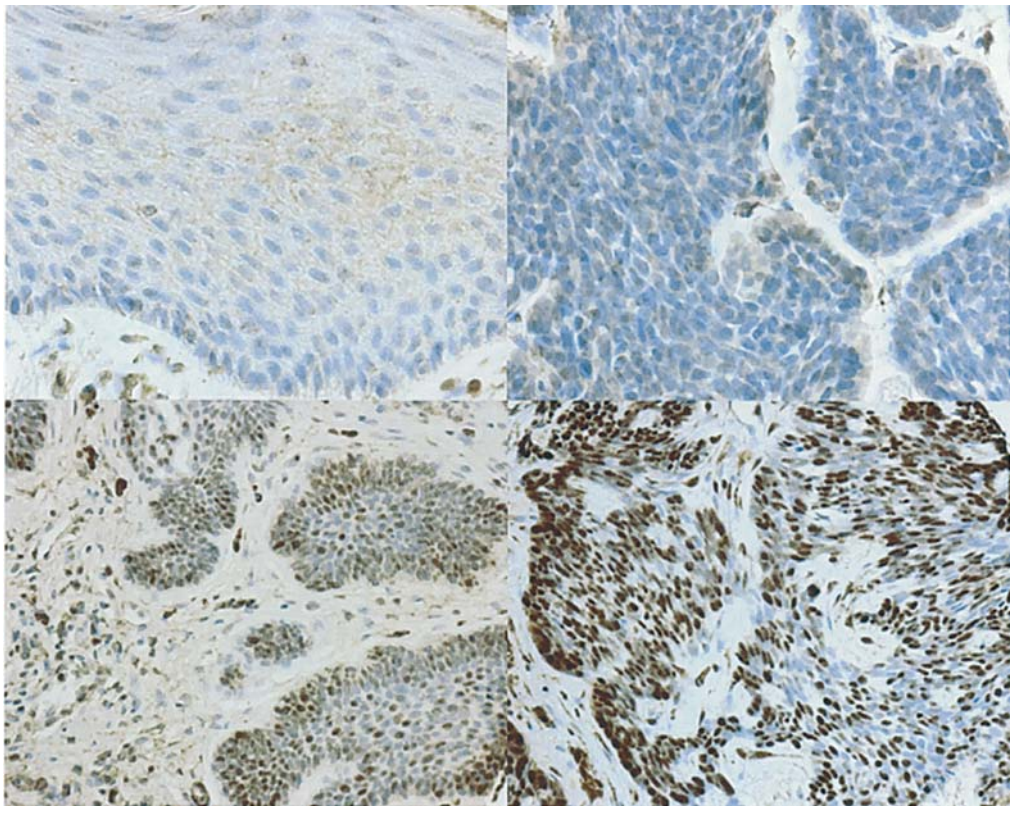

Figure 1. Differential Nmyc protein expression in BCC and normal skin. Detection of differential Nmyc protein expression on TMA sections. Normal skin (top left) exhibits no Nmyc staining. For BCC specimens, 3 examples of no (-; top right), weak (+; bottom left) and strong (++; bottom right) Nmyc expression are shown. Original magnification, x40 (top panels) and x20 (bottom panels).

and NF-κB pathway genes (1), which were found to be overexpressed in BCC. Nevertheless, the detailed pathogenic mechanisms and possible co-factors of these genes in inducing BCC formation are little understood.

Studies on medulloblastoma, a primitive neuroectodermal childhood malignant brain tumour arising from cerebellar granule neuron precursors, identified *NMYC* as a direct target gene of the Shh pathway, suggesting that Nmyc protein is an important mediator of Shh-induced proliferation and tumourigenesis (9,10). *NMYC*, a member of the MYC family, encodes a basic helix-loop-helix leucine zipper protein that is supposed to primarily serve as a transcriptional activator (11). The role of *NMYC* in tumour formation has been extensively investigated in neuroblastoma (12) and medulloblastoma (13), but no such association has been determined yet for BCC. To elucidate the role of the Shh downstream effector, Nymc, in BCC pathogenesis, we analysed a representative collection of 273 BCC specimens on a tissue microarray (TMA) for Nmyc expression by immunohistochemistry and for *NMYC* gene copy number changes by fluorescence *in situ* hybridisation (FISH).

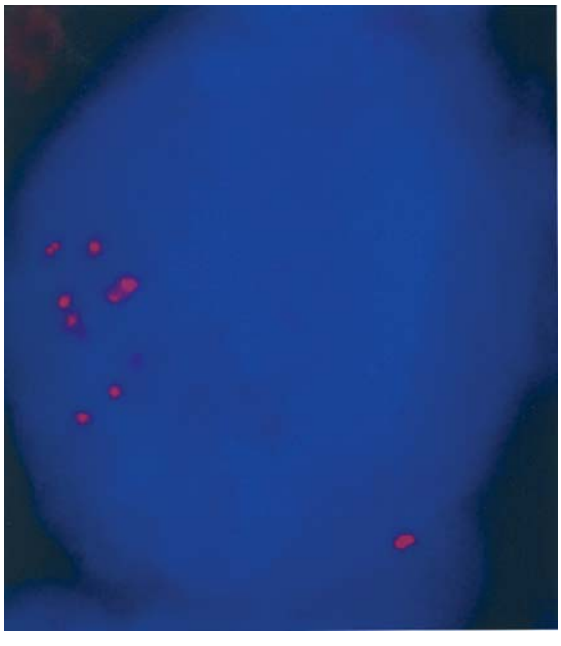

Figure 2. *NMYC* amplification in a BCC nucleus. Example of a BCC tumour cell exhibiting distinct *NMYC* gene amplification. Eight to nine FISH signals (red) can be observed inside the nucleus of the tumour cell (blue).

Materials and methods

Generation of tissue microarrays (TMA). TMA construction was performed as described previously (14). Briefly, tissue cylinders with a diameter of 0.6 mm were punched out of the donor block and applied to a recipient block using a tissue microarrayer (Beecher Instruments, Silver Spring, MD). The recipient block was cut into 5-μm sections using standard techniques. A total of 273 basal cell carcinomas (BCC) from 242 patients were available on the BCC-TMA. The tumours

Table I. IHC results of Nmyc protein analysis on TMA sections.

IHC staining	Negative (-)	Weak (+)	Strong (++)
BCC subtype			
Nodular (n=146)	44	55	47
Superficial (n=15)	3	7	5
Infiltrative (n=59)	13	15	31
All subtypes (n=220)	60	77	83
Anatomic site			
Head (n=165)	41	52	72
Trunk/extremities (n=31)	10	14	7
All BCC (n=196)	51	66	79

Summary of IHC results obtained from TMA analysis in different growth patterns (above) and anatomic localisation (below) of BCC.

Table II. FISH results of *NMYC* analysis on TMA sections.

FISH result	ba (n)	cng (n)	cng (%)
BCC subtype			
Nodular (n=135)	108	27	20.0
Superficial (n=11)	9	2	18.0
Infiltrative (n=65)	57	8	12.0
All subtypes (n=211)	174	37	17.5
Anatomic site			
Head (n=169)	139	30	18.0
Trunk/extremities (n=26)	21	5	19.0
All BCC (n=195)	160	35	18.0

Summary of *NMYC*-FISH results (ba, balanced locus; cng, copy number gain) obtained from TMA analysis in BCC of different growth patterns (above) and anatomic localisation (below).

were subdivided according to their predominant histological growth pattern (nodular, superficial or infiltrative). As controls, 8 specimens of two unaffected skin biopsies from healthy donors were also included in the BCC-TMA.

Fluorescence in situ hybridisation (FISH) on TMA sections. FISH experiments were performed as described elsewhere (14). *NMYC* FISH probe directly labelled with rhodamine was hybridised together with a chromosome 2 α-satellite probe labelled with fluorescein as control (Qbiogene, Wiesbaden, Germany). At least 50 cells were counted per individual core for the number of nuclear FISH signals. *NMYC* copy number gain was scored if >30% of all cells analysed showed three or more FISH signals. If at least 10% of all cells analysed also obtained six or more FISH signals, a specific high-level gene amplification of *NMYC* was defined. The signals of centromeric probes were used to control adequate hybridisation and exclude artefacts.

Immunohistochemistry (IHC) on TMA sections. Mouse monoclonal anti-Nmyc antibody (Calbiochem, Darmstadt, Germany) was used at a dilution of 1:100. As a detection system, the Dako catalysed signal amplification (CSA) horseradish peroxidase system (Dakopatts; Copenhagen, Denmark) was applied according to the instructions of the manufacturer, including microwave treatment. Immunoreactivity was visualised with 3'3'-diaminobenzidine as the chromogen (15). For the semiquantitative evaluation of IHC experiments, nuclear staining was taken into account. Tumours with <10% positively stained cells were considered negative (-), whereas tumours with 10-50% positively stained cells were considered weakly positive (+) and those with >50% were strongly positive (++).

Statistical analysis. The prevalence of *NMYC* copy number gains in FISH analysis and the distribution of different staining intensities by IHC analysis in BCC subgroups were analysed using Fisher's exact test. P-values ≤ 0.05 were considered significant.

Results

Nmyc protein expression was analysed by IHC in 220 BCC specimens on TMA sections. Of all tumours analysed, 72.7% (160/220) exhibited a positive staining score (+/++), whereas all control specimens were scored as negative (-). To define the extent of Nmyc immunoreactivity in different histological subgroups, the prevalence of differential Nmyc expression in the less aggressive nodular and superficial subtypes was tested versus the more aggressive infiltrative subtype (16,17). It was shown that strong (++) Nmyc immunopositivity was more frequently found in infiltrative BCCs compared to nodular/superficial BCCs (p=0.005). Furthermore, BCCs with available anatomic localisation were tested for differential Nmyc expression (head vs. trunk/extremities). Of note, strong (++) Nmyc immunopositivity was found more frequently in BCCs of the head than in tumours of other anatomic localisations (p=0.021). All data obtained by IHC analysis are summarised in Table I. Examples for different categories of immunostaining are presented in Fig. 1.

To assess whether an elevated *NMYC* gene copy number contributes to increased Nmyc protein expression, FISH analysis of the *NMYC* gene locus was performed on BCC-TMA sections for 211 BCCs. The prevalence of *NMYC* copy number gains was 17.5% (37/211), including three tumours with nodular differentiation that exhibited a distinct high-level amplification of the *NMYC* locus (Fig. 2). No statistical difference was found between the prevalence of *NMYC* copy number gains in nodular/superficial BCC versus infiltrative BCC (p>0.05). All data obtained by FISH analysis are summarised in Table II.

For 159 BCC specimens, FISH and IHC data were available. To analyse whether an *NMYC* copy number gain results in protein overexpression in individual TMA cores, tumours inheriting a *NMYC* copy number gain were compared to tumours with a balanced *NMYC* locus for Nmyc protein expression. Though the frequency of tumours with strong Nmyc protein expression (++) was higher in BCCs with an *NMYC* gene copy number gain (13/27, 48.1%) than BCCs

with a balanced *NMYC* locus (59/132, 44.7%), there was no statistical difference between these two groups ($p>0.05$).

Discussion

Amplifications of the oncogene, *NMYC,* were initially found in neuroblastoma cell lines, a highly malignant embryonal tumour of childhood (18). Located on chromosomal band 2p23-24, *NMYC* was detected inside tumour-specific chromosomal abnormalities like double minute chromosomes (dm) and homogenously staining regions (hsr) (19). In primary neuroblastomas, *NMYC* amplifications correlate with advanced tumour stage and adverse patient outcome (12). NMYC amplifications were observed in a variety of further tumour entities such as small cell lung cancer, retinoblastoma, malignant gliomas (20) and rhabdomyosarcomas (21).

In the present study, copy number gains and a small number of distinct high-level amplification of *NMYC* were found in BCC, indicating that these genetic aberrations might contribute to BCC development by consecutive high expression of the Nmyc protein. In most other tumour entities, increased Nmyc mRNA and protein expression were the result of a 50- to 100-fold *NYMC* amplification. For neuroblastoma, duplication of the *NMYC* locus was also described, suggesting an alternative mechanism of *NMYC* activation by low level gene copy number gain (22). This mechanism might be active in BCC. However, the prevalence of *NMYC* aberrations, as detected by FISH analysis on TMA sections, was <20% in this study and did not approach the estimated frequency of >70% Nmyc high expression as assessed by IHC in the same tumour collection. Furthermore, the prevalence of a BCC specimen exhibiting strong Nymc expression was only slightly higher in tumours with an increased gene copy number compared to the group of tumours with a balanced genotype, suggesting that further molecular mechanisms in addition to gene copy number gain contribute to the high prevalence of increased Nmyc expression in BCC. High Nmyc expression without amplification of the gene had been observed in retinoblastoma cell lines and Wilms tumour specimens (23). Since the mechanism remained unclear, and increased Nmyc expression was also detected in various embryonic cells (including retinal and kidney cells) and the human foetal brain, it was assumed that increased Nmyc expression in tumours, which do not carry an amplification, might be a normal feature of undifferentiated cells and merely reflect the differentiation state of the tumour (20). Interestingly, however, studies on cultured cerebellar granule neuron precursors (CGNPs) found that Nmyc is a direct target molecule of the Shh pathway in promoting cell cycle progression in cerebellar development. Furthermore, increased Smo-independent Nmyc expression was observed in medulloblastomas of Ptch-deficient mice, indicating that Nmyc acts as an Shh downstream effector not only in physiological differentiation, but also in malignant transformation (9). With regard to the frequent Nmyc high expression found in this study, one could therefore argue that this is caused by gene copy number gains only in a subset of BCC specimens, while increased protein expression might result from aberrant Shh signalling in the majority of cases.

Several molecular factors were supposed to be involved in the emergence of different BCC subtypes. For example, the anti-apoptotic gene, bcl-2, shows markedly reduced expression in the infiltrative subtype compared to the nodular and superficial subtype (16). For E-cadherin, a similar association of reduced expression and infiltrative BCC subtype was found (24). Interestingly, we detected a higher prevalence of strong Nmyc expression in BCC with an infiltrative growth pattern. This result further supports the hypothesis that different molecular pathways contribute to the formation of BCC subtypes.

UV-induced *PTCH1* mutations are directly involved in BCC pathogenesis (25). Most BCCs occur on sun-exposed regions of the head, whereas anatomic regions like the trunk and extremities are less frequently affected. Nevertheless, the significant number of BCCs arising on unexposed or less sun-exposed areas of the body suggest that not only are UV-induced *PTCH1* mutations responsible for BCC development, but other factors as well (26). Our finding that the Shh-mediator, Nmyc, more frequently showed strong expression in cranial BCC than in tumours of the trunk and extremities supports the thesis that aberrant Shh signalling is predominantly involved in sun-exposed BCC, and suggests different molecular pathways in non-sun-exposed BCC.

In most primary BCCs, surgical excision is still the predominant standard therapy, which usually leads to a complete removal of the tumour (27). However, in advanced and recurrent BCC, and in BCC patients suffering from Gorlin's syndrome with a high prevalence of BCCs during their lifetime, mutilating resection approaches must frequently be performed to obtain clear surgical margins. Novel non-invasive therapy forms like photodynamic therapy (28) or topical application of 5-fluorouracil (29) and imiquimod (30) have shown promising results in BCC treatment. However, as the tissue penetration rates of the applied agents are currently poor, only superficial lesions are successfully treated, making these options of limited value for the treatment of advanced and recurrent BCC. The finding of an *NMYC* gene copy number gain and high protein expression in BCC might offer novel therapeutic options in inhibiting Nmyc-mediated signalling. In a murine transgenic neuroblastoma model system, it was demonstrated that decreased expression of the Nmyc protein can be achieved by using systemically applied *NMYC* antisense oligonucleotides (31). Furthermore, peptide nucleic acids (PNA) that belong to the most recent generation of nucleic acid therapeutics were previously utilised in experimental anti-*NMYC* approaches, allowing more effective inhibition of gene expression, since they form highly stable complexes with target RNA and are resistant to degradation by nucleases and proteases (32). Our results suggest that Nmyc antisense therapy, which is currently being investigated for use in the treatment of childhood neuroblastoma, may be clinically useful for the therapy of BCC, particularly for those with advanced or recurrent tumours who experience local therapy failure of standard treatment approaches.

In conclusion, TMA analysis revealed frequent high protein expression of the Shh downstream mediator, Nmyc, in BCC, predominantly detected in lesions exhibiting an infiltrative growth pattern. *NMYC* copy number gains found in a subset of cases might contribute to aberrant Nmyc protein expression by a gene dosage effect. In the light of novel systemic antisense *NMYC* inhibition strategies, targeted *NMYC* therapy

could be a promising option for therapy refractory BCC, which should be investigated in future clinical trials.

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