Asian Biomedicine Vol. 1 No. 1 June 2007

Review

Chromogenic in situ hybridization using routine tissue sections: *MYCN* in neuroblastoma

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Background: Amplification of the *MYCN* (myc myelocytomatosis viral related oncogene, neuroblastoma derived) gene in neuroblastoma is associated with a poor prognosis. Methods for estimating *MYCN* gene copy number that are based on pooled cells do not address copy number heterogeneity at the cell level and can underestimate or even miss amplification. *MYCN* copy number can be directly assessed by fluorescence in situ hybridization, but evaluation of tissue histology is difficult if not impossible.
Objective: This paper reviews chromogenic in situ hybridization (CJSH) as it applies to the *MYCN* gene in neuroblastoma. We compare this technique to other methods for determining gene copy number and highlight the advantages of CISH.
Methods: We have developed a chromogenic method for in situ hybridization (CISH) that enables us to determine *MYCN* copy number on an individual cell basis. This technique uses light microscopy on routine paraffin sections, and therefore allows simultaneous assessment of tumour histology.
Results: In a previous study, CISH identified 100 % of the cases that were known to be amplified by other techniques and proved to be more sensitive than Southern blotting or the quantitative DNA polymerase chain reaction. The *MYCN* copy number is generally believed not to vary within a tumour, nor between tumour samples, including primary vs. metastases, and pre-and post-treatment specimens. However, we found heterogeneity from cell to cell, with ~30 % of amplified tumours showing >50 % variation in *MYCN* copy between cells.
Conclusion: For detection of gene amplification, CISH has all the advantages of FISH but in addition, needs no special microscopy or image capturing systems, and preparations are permanent. In the case of neuroblastoma, CISH has disclosed considerable heterogeneity in *MYCN* copy number between cells in a tumour. Heterogeneity reflects different tumour clones and its role has been under-recognized in neuroblastoma biology. Additional studies are needed to investigate the significance of tumour heterogeneity in neuroblastoma, and whether the aggressive (i.e., *MYCN*-amplified) clones are more likely to metastasize, survive treatment modalities, and ultimately kill the patient.

Keywords: CISH, FISH, *MYCN*, neuroblastoma, tumour heterogeneity.

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Neuroblastoma is the fourth most common malignancy of childhood (after brain tumours, leukemia and lymphoma). This tumour is a malignancy of neural crest cells of the autonomic nervous system, hence it is typically found in the adrenal medulla or sympathetic ganglion chain [1]. It occurs most frequently in children less than 5 years of age and accounts for 15 % of cancer-related deaths in childhood [2]. Neuroblastoma, along with retinoblastoma and rhabdomyosarcoma, is one of the few tumours characterized by the occurrence of amplification of a particular oncogene, *MYCN* (myc myelocytomatosis viral related oncogene, neuroblastoma derived) [3, 4]. Gene amplification refers to an increase in the relative number of copies of a particular gene per cell. In neuroblastoma, this can range from one or two additional copies per cell to over a hundred copies per cell. The term 'relative increase' is important, since polysomy such as triploidy or tetraploidy do not represent amplification. Amplification is believed to result in over-expression of critical genes involved in oncogenesis.

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The *MYCN* gene maps to chromosome 2p23-24 and is a member of a family of *MYC* genes and its expression is normally restricted to neural tissues during embryogenesis [5,6]. Increased copies of *MYCN* generally lead to increased transcriptional activity of target genes, only some of which are known. Amplification of the *MYCN* gene confers a growth advantage to cells *in vitro* [7]. There is also a transgenic mouse model that overexpresses *MYCN* in neuroectodermal cells [8]. This mouse develops neuroblastoma confirming that *MYCN* contributes to the oncogenesis of this tumour, although the exact sequence of genetic events is still unknown.

When the *MYCN* gene is amplified, it usually takes the form of double minute chromosomes, separate from the normal 46 chromosomes, and visible in cytogenetic preparations. Less often, the extra copies become integrated as tandem repeats into some random site in a chromosome. This can be recognized as a homogeneously staining region in a cytogenetic spread [3, 9, 10]. Approximately 25 % of neuroblastomas show amplification of the *MYCN* gene [11] but this is more common (about 40 %) in high stage tumours (stage 3 and 4) and uncommon (5-10 %) in low stage tumours (stage 1, 2, or 4S) [12, 13].

Greater than 10 copies of the *MYCN* gene is an independent poor prognostic indicator, and is associated with advanced tumour stage, rapid tumour progression, and poor outcome, regardless of tumour stage [11, 12, 14, 15]. A lack of *MYCN* gene amplification does not necessarily imply a good prognosis. It is well known that high stage neuroblastomas without *MYCN* amplification also have a poor outcome although the molecular basis for this is poorly understood. The significance of *MYCN* gene amplification in low stage tumours is less straightforward Some have found that this still confers a poor prognosis regardless of low stage [16], while others have found that patient outcome was better predicted by histology rather than *MYCN* amplification status [17].

Determination of *MYCN* copy number

While amplified *MYCN* genes can be seen in traditional cytogenetic spreads either as double minute chromosomes or homogeneously staining regions, this method is too slow and expensive for routine clinical use. Instead, *MYCN* copy number was originally determined by Southern blotting [11] and later other methods such as quantitative DNA polymerase chain reaction (PCR) were introduced [18]. These methods analyze DNA pooled from a mixture of cells and therefore provide only an average result for a particular tumour. When the result is >10, this presents no problem in determining patient prognosis and planning treatment. However, when the copy number is in the 3-10 range, it is unclear what this means. Such a result could be obtained from (1) a heterogeneous population of tumour cells in which a small proportion are highly amplified, (2) a highly amplified tumour mixed with normal tissue, or (3) a uniform low level of increased copies in tumour cells (e.g. a triploid population). The first two situations carry poor prognosis, whereas triploid neuroblastomas have a favorable prognosis, yet all would be regarded as non-*MYCN*-amplified for treatment purposes. Because key therapeutic decisions are based on the presence of *MYCN* amplification, physicians treating children with neuroblastoma need to be aware of the possibility that *MYCN* amplification may be heterogeneous within a tumour and may be missed using techniques based on pooled DNA such as Southern blotting.

These difficulties have been overcome to a large extent by using fluorescent in situ hybridization (FISH) to determine *MYCN* copy number [19-23]. FISH has many advantages in the clinical setting:

(1) FISH is as sensitive as Southern blotting or quantitative PCR;
(2) FISH requires a smaller sample size than needed for Southern blotting;
(3) FISH has a faster turnaround time than Southern blotting or traditional cytogenetic analysis;
(4) FISH can be used on interphase nuclei, in contrast to karyotype analysis that requires dividing cells;
(5) FISH can be applied to formalin-fixed nuclei, in cases where fresh or frozen tumour is not available;
(6) FISH can distinguish double minute chromosomes from a homogenously staining region, but this has yet to see any clinical application;
(7) FISH can identify amplified cells within a mixture of amplified and non-amplified cells.

However, FISH also has disadvantages that are inherent to fluorescent microscopy:

(1) specialized, expensive equipment is required for viewing slides;
(2) tumour cells preparations are not permanent;
(3) storage of results means that images must be

captured, which requires specialized cameras and software;
(4) since tumour cells are dissociated from each other before examination by FISH, information about tissue architecture is lost;
(5) it is difficult if not impossible to distinguish normal cells from tumour cells.

The disadvantages of FISH mentioned above can all be overcome if the detection system is converted to the light microscopic level. This is the principle behind *chromogenic in situ hybridization* (CISH). We have established a CISH method that allows determination of *MYCN* copy number in formalin-fixed paraffin-embedded tissue sections [1]. To date, three other studies have also used CISH to determine *MYCN* copy number [24-26]. We will review this technique in comparison to Southern blotting, quantitative PCR and FISH, plus point out the advantages this technique offers over these other approaches. We have found the technique to be simple, easy to interpret and applicable to routinely processed specimens, including archival material. We feel that this technique could be used routinely for evaluation of *MYCN* copy number in neuroblastoma.

MYCN CISH protocol for formalin-fixed paraffin-embedded tissue sections

Five micron formalin-fixed paraffin-embedded sections are mounted on positively charged glass microscope slides and baked in a 60 °C oven for 2-3 hours. Tissue sections are then deparaffinized and rehydrated through graded alcohols. Tissue sections are then boiled at 98 °C for 15 minutes in CISH Tissue Heat Pretreatment Solution (Zymed Laboratories, San Francisco, USA), washed in distilled water, followed by treatment with an Enzyme Pretreatment Reagent (Zymed) at room temperature for 10 minutes. The tissue sections are then washed in distilled water, dehydrated through increasing concentrations of alcohol to absolute alcohol, and air-dried. 15-20 µl of *MYCN* double-stranded DNA probe (Zymed) labelled with digoxigenin is applied to the tissue sections, coverslipped and sealed with a rubber solution. Denaturation is performed at 95 °C for 5 minutes followed by hybridization overnight at 37 °C in a HYBrite thermal incubator (Vysis Inc, Downers Grove,IL, USA). After hybridization, the coverslips are removed from the tissue sections and the slides rinsed in 0.5x SSC at room temperature followed by 5 minutes in 0.5x SSC at 75 °C. Signal detection is performed using the SPOT-LIGHT CISH Polymer Detection Kit (Zymed) as described in the manufacturer. This includes incubation with a mouse polymerized anti-digoxigenin and horseradish peroxidase-goat anti-mouse sequence followed by exposure to DAB chromogen. After a light hematoxylin counterstain, the sections are dehydrated, cleared in xylene and coverslipped. Normal tissue in the section provides an internal control for hybridization and a highly amplified neuroblastoma is run as a positive control with each assay.

Scoring of tissue sections for _MYCN_ copy number by CISH

Tumour cells can be easily distinguished from normal cells since one is looking at a routine section. At least 200 tumour nuclei should be evaluated in each case. Tumour cell nuclei often overlap, which could lead to errors in copy number determination; hence, care should be taken to score only non-overlapping cells. Tumours are scored as ‘not amplified’ if there are only two hybridization signals per nucleus (**Fig. 1**). Occasionally, only one signal is seen, which can be attributed to sectioning with loss of nuclear material. Cells undergoing mitosis might be expected to have 4 signals per nucleus and such cells are not scored, partly because they cannot be easily distinguished from signals present in overlapping nuclei. We found a few cases with 3 copies of the *MYCN* gene per nucleus and considered these were likely triploid tumours.

In our experience, cases that are amplified can usually be identified even at lower magnifications, but counting hybridization signals requires a 60x objective or higher. Tumours with *MYCN* amplification show varying numbers of signals. In most cases, these cannot be counted accurately since the signals overlap within individual nuclei. This is particularly true when there are >25 signals per nucleus. However, since any number of signals greater than 10 per nucleus is scored as ‘amplified’, is it not necessary to arrive at an exact count for the number of *MYCN* signals in each nucleus. It is also possible to distinguish two staining patterns by CISH (**Fig. 2**). Most cases show the pattern expected for double minute chromosomes with numerous signals evenly distributed throughout the nuclei. A rare case will show the pattern for a homogeneously staining region with a large aggregate of signal within the nucleus.

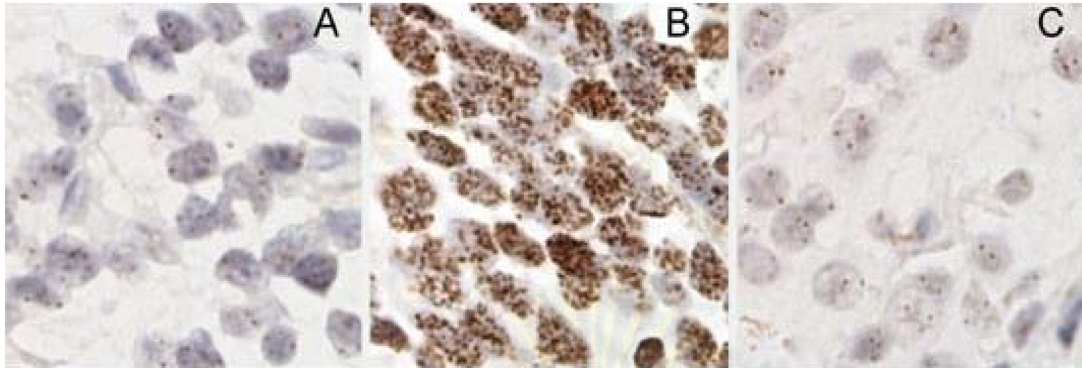

Fig. 1 *MYCN* copy number in neuroblastoma by CISH. (**A**) A tumour that is diploid for *MYCN* with two signals per nucleus. (**B**) A tumour that is amplified for *MYCN* with >25 signals per nucleus. (**C**) A tumour that is triploid for *MYCN* with 3 signals per nucleus. (CISH with DAB and hematoxylin counterstain; original magnification, 40X).

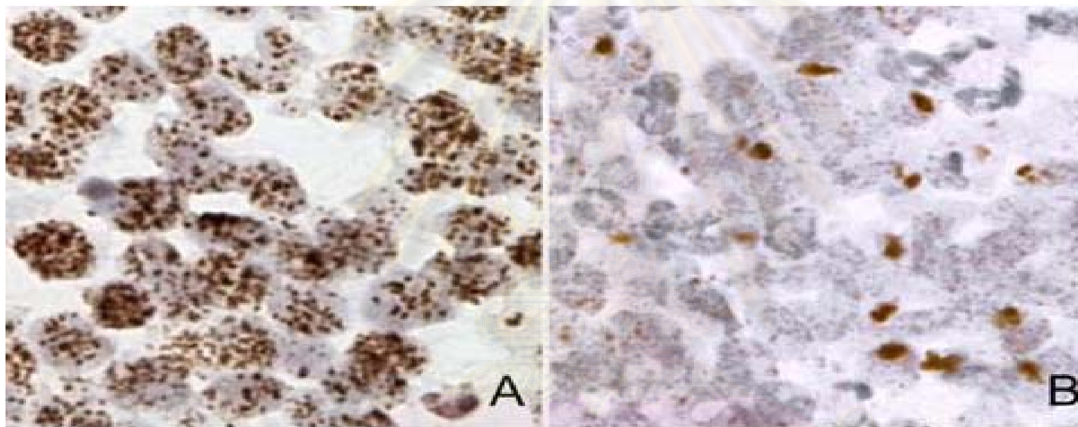

Fig. 2 Nature of amplified *MYCN* genes in neuroblastoma. (**A**) A tumour with numerous discrete signals throughout the nuclei indicating the presence of double minute chromosomes. (**B**) A tumour with large aggregates of signals, indicating the presence of homogeneously staining regions. (CISH with DAB and hematoxylin counterstain; original magnification, 40X).

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CISH can easily be set up in laboratories not equipped to do FISH or molecular genetic studies. The scoring of sections for *MYCN* copy number is straightforward and does not require any labour-intensive quantitation or scoring systems. We experienced no problems separating neuroblastoma cases into 'amplified' and 'non-amplified' using CISH. Other studies [24-26] have found similar results, although one of these studies used 6 or more signals per nucleus as indicative of amplification. We think this could lead to some patients being called amplified that are not and recommend keeping the standard of >10 signals per nucleus as the cutoff value for amplification.

Comparison to other methods of *MYCN* copy number determination

In our study CISH identified 100 % of cases of neuroblastoma that were previously known to be amplified by Southern blotting, quantitative PCR or FISH [1]. FISH also identified all cases, whereas Southern blotting missed 3 cases and quantitative PCR missed 2 cases. Thus, CISH was as reliable as FISH for categorizing tumours as 'amplified' or 'non-amplified' and more accurate than Southern blotting or PCR. Others studies have reported similar results for CISH with a concordance of >93 % with Southern blotting and 100 % correlation with FISH [25,26] The copy number determined by CISH agreed more closely with the FISH results than the other two

methods [1]. However, the CISH copy number was often lower than FISH copy number. This can be attributed to two factors: first, sectioning of nuclei in paraffin sections reduces the number of *MYCN* genes per nuclear cross section; and second, the DAB amplification step makes visual separation of signals more difficult than with FISH, especially when there are >25 signals per nucleus.

Low levels of *MYCN* amplification

Both Southern blotting and PCR rely on pooled DNA samples, which can include non-neoplastic tissue that can dilute out *MYCN*-amplified cells, leading to underestimation of *MYCN* copy number. The concept of dilution of tumour by normal cells is of concern for tumours in the category of low level of *MYCN* amplification (3-9 copies per cell), and specifically whether these copy numbers are present in all the tumour cells (a good prognosis), or does this result reflect a population of amplified cells diluted out by normal cells (a poor prognosis).

To ask if aneuploidy can account for results in this range, we correlated our CISH results with flow cytometry results. In our study [1], ~20 % cases showed low levels of amplification by CISH, and half were aneuploid by flow cytometry and half, near diploid. Thus, low increases in *MYCN* copy number can sometimes be attributed to aneuploidy but not in all cases. The other cases likely had increased numbers of diploid cells in the S or G2M phase, such that signals of 3 or 4 were more common than in other tumours. There were also cases that were aneuploid, triploid or tetraploid on flow cytometry, but with only two copies of *MYCN* per nucleus by CISH. This may be related to sectioning of tumour nuclei reducing the number of signals available for detection, but some cases also had two *MYCN* signals by FISH, in which whole nuclei are examined. The alternate explanation is that the aneuploidy noted by flow cytometry did not involve chromosome 2, hence, and thus there was no increase in the *MYCN* copy number. It should also be pointed out that flow cytometry cannot be used to quantitate *MYCN* copy number since the amount of DNA contributed by the extra copies of *MYCN* does not significantly alter the DNA index.

Nucleolar size and correlation with *MYCN* copy number

It has been reported that increased nucleolar area correlates with amplification of the *MYCN* gene in stroma-poor neuroblastoma [27]. This observation was based on correlating the measured area of the nucleolus in a tissue section with the *MYCN* copy number determined by FISH. The study found that *MYCN*-amplified tumours had a larger nucleolar area than non-amplified tumours. Rather than perform detailed morphometry, we designated nucleoli as 'macronucleoli' if they resembled the appearance typically seen in rhabdoid tumours. By this definition, we found ~15 % of neuroblastoma cases had enlarged nucleoli, and surprisingly, 100 % of these had *MYCN* amplification by CISH (**Fig. 3**). On the other hand, there were more cases with *MYCN* amplification that did not have such nucleoli [1]. Hence the specificity for this morphologic finding would seem to be high, but the sensitivity is low, a finding that was also noted in the original study [27].

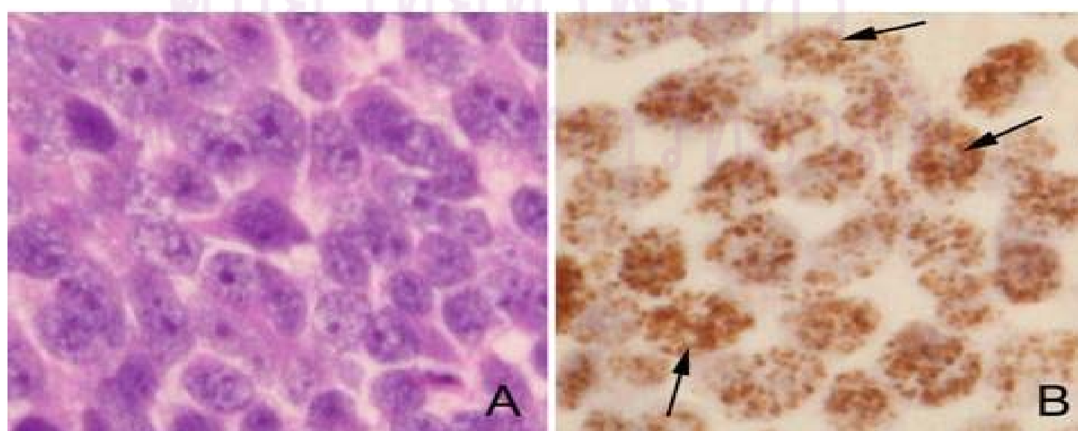

Fig. 3 Macronucleoli in neuroblastoma. Tumours with large 'rhabdoid tumour' size nucleoli (**A**) have *MYCN* amplification (**B**), although most amplified cases do not show this nucleolar morphology. Arrows mark the nucleoli, which are not sites of extra *MYCN* copies. (**A**: hematoxylin and eosin; B: CISH with DAB and hematoxylin counterstain; original magnification, 60X).

Heterogeneity of *MYCN* copy number within a tumour sample

The copy number for *MYCN* is believed to remain constant between concurrent and consecutive samples from a patient [28], but this concept is based on Southern blotting that measures only an average copy number in a sample. Using FISH, it has been shown that there is considerable heterogeneity in the number of *MYCN* signals per cell [19, 20, 22, 23]. This is thought to be a result of unequal segregation of double minute chromosomes between daughter cells at mitosis, resulting in a heterogeneous distribution of double minute chromosomes over time as the neuroblastoma proliferates. Our previous work using FISH found that ~17 % of tumours showed heterogeneity for *MYCN* copy number [29], which is in direct conflict with the concept that *MYCN* copy number is consistent within a particular tumour. We felt that 17 % was likely an underestimate, since it can be difficult when using FISH to distinguish between tumour and normal tissue, and foci of tumour with low levels of amplification could easily be passed off as normal tissue.

CISH overcomes this limitation since the sections are examined by light microscopy. We found cases with increased copies of *MYCN* usually showed similar numbers of signals from nucleus to nucleus, although there was some variation in the absolute number of signals per nucleus. We did not score these differences as 'heterogeneity of *MYCN* copy number' since we could not rule out that the variation in number was related to sectioning of nuclei combined with the difficulty in obtaining exact counts in cases with >25 signals per nucleus. To avoid this problem, we defined 'heterogeneity of *MYCN* copy number' as a 50 % or greater difference in copy number from cell to cell (**Fig. 4**). Using this definition, we found that ~29 % of neuroblastomas studied showed heterogeneity of *MYCN* copy number [1]. Some of these cases had been reported as non-*MYCN* amplified by Southern blotting and/or PCR, but those cases that had FISH done also detected heterogeneity. This proportion of heterogeneity is higher than our previous study and we attribute this to using CISH instead of FISH. It should be mentioned, however, that studies using tissue microarrays [25, 26] examine only small amounts of tumour and will probably miss or underestimate the degree of *MYCN* heterogeneity.

Heterogeneity of *MYCN* copy number between tumour samples

It is not unusual for the histology of neuroblastoma to vary within a sample and between samples, reflecting differences in maturation. Neuroblastoma often shows some maturation following chemotherapy or spontaneously. It is not uncommon for the primary and the metastasis to differ in histology, with the metastasis less differentiated, and different metastases sometimes have different histologies. Nevertheless, it is generally believed that *MYCN* amplification is homogeneous within a tumour pre- and post-treatment, and congruent between the primary tumour and metastases [28]. We have shown that during the course of treatment, there was continued growth of

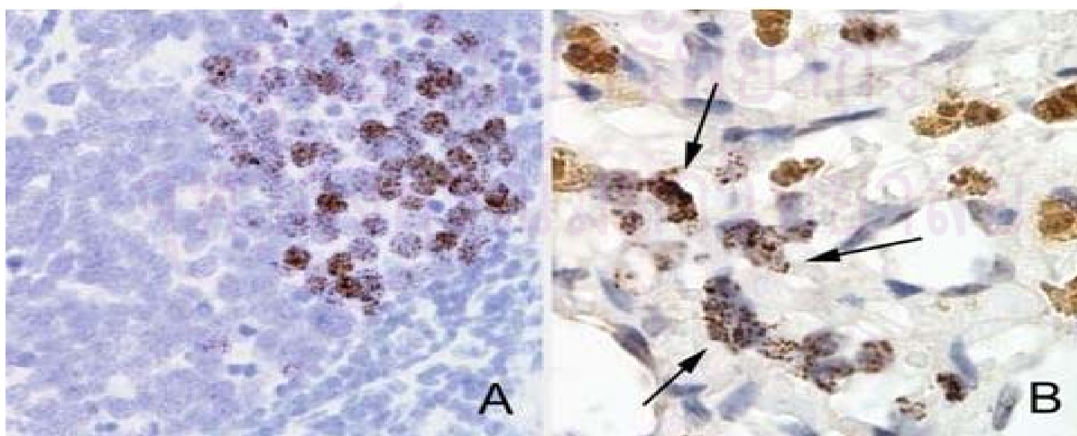

Fig. 4 Variability in number of *MYCN* signals. (**A**) A case with heterogeneity of *MYCN* copy number with a population of *MYCN*-amplified tumour cells within a background population of non-amplified tumour cells. (**B**) CISH is also useful to identify residual islands of *MYCN*-amplified tumour cells (arrows) within a background of scar tissue and hemosiderin-laden macrophages. (CISH with DAB and hematoxylin counterstain; original magnification, 40X).

the *MYCN*-amplified component in a neuroblastoma that was originally heterogeneous with respect to *MYCN* copy number [30]. This clonal overgrowth changed the tumour from a favourable prognostic category to an unfavourable one. Thus, at least some cases of neuroblastoma contain genetically distinct clones that differ by *MYCN* copy number. Support for the concept of clones with different biologic potential also comes from our study of telomerase activity in neuroblastoma [31]. Telomerase expression is an unfavourable prognostic indicator [32]. In our study, tumours were assessed pre- and post-chemotherapy, and ~25 % showed higher telomerase activity after therapy, consistent with overgrowth of a more aggressive clone of neuroblastoma cells.

Since *MYCN* amplification is a marker of aggressive disease, these different clones could have different biologic behaviour, which in turn could impact on response to treatment and overall survival. CISH is an ideal way to identify these clones in patient biopsies. Since *MYCN* heterogeneity is an under recognized phenomenon, its clinical significance remains to be determined. A larger study is needed to address the extent of *MYCN* heterogeneity in neuroblastoma, and correlate this with clinical outcome and pathologic parameters such as tumour differentiation. Such a study would need to include tumour samples from the primary, pre-and post-treatment, as well as tumour metastases. Treatment may select for resistant clones that are *MYCN*-amplified. As well, since metastases are generally individual clones from the primary, they may carry a higher *MYCN* copy number than the primary, especially if there is *MYCN* heterogeneity within the primary.

Conclusion

CISH technology allows a reliable determination of *MYCN* copy number in formalin-fixed paraffin-embedded specimens. It is as accurate as FISH for categorizing neuroblastoma as ‘amplified’ or ‘non-amplified’ and offers several advantages over FISH:
(1) CISH utilizes routine light microscopy;
(2) the slide preparations are permanent;
(3) no special storage of slides is required;
(4) immediate capturing of images to record results is not needed;
(5) tissue architecture can be evaluated in combination with *MYCN* copy number.

The ability to interpret tissue architecture avoids the problem of counting normal cells and allows one to score small populations of surviving tumour within large necrotic areas. CISH is thus an attractive method for any lab handling neuroblastoma specimens, especially for institutions not set up to handle fresh or frozen tissue or carry out molecular genetic studies, since the technique is very similar to immunohistochemistry that almost all pathology labs have available. The ability to examine cellular and tissue morphology also allows for studies that have been impossible previously, since CISH allows analysis of *MYCN* copy number on an individual cell basis. We have shown that *MYCN* copy number can be heterogeneous in neuroblastoma and thus the concept that *MYCN* amplification is homogeneous within a tumour and congruent between primary tumour and metastasis may be an oversimplification. CISH opens the possibility to correlate *MYCN* copy number with other morphologic parameters such as tumour differentiation, and follow changes in copy number before and after treatment, in both primary and metastatic lesions. Such studies will lead to a better understanding of neuroblastoma tumour biology. Finally, on a larger perspective, *MYCN* in neuroblastoma is just one example of the application of CISH and the types of studies possible. Similar approaches could be taken for any cancer in which gene amplification plays a role and for which a suitable in situ probe is available.

Acknowledgement

This work was supported by the James Birrell Neuroblastoma Research Fund, Hospital for Sick Children, Toronto, Canada.

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