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# Immunohistochemical evaluation of molecular radiotherapy target expression in neuroblastoma tissue

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## Abstract

### Purpose

Neuroblastoma may be treated with molecular radiotherapy,  $^{131}\text{I}$  meta-Iodobenzylguanidine and  $^{177}\text{Lu}$  Lutetium DOTATATE, directed at distinct molecular targets: Noradrenaline Transporter Molecule (NAT) and Somatostatin Receptor (SSTR2) respectively. This study used immunohistochemistry to evaluate target expression in archival neuroblastoma tissue, to determine whether it might facilitate clinical use of molecular radiotherapy.

### Methods

Tissue bank samples of formalin fixed paraffin embedded neuroblastoma tissue from patients for whom clinical outcome data were available were sectioned and stained with haematoxylin and eosin, and monoclonal antibodies directed against NAT and SSTR2. Sections were examined blinded to clinical information and scored for the percentage and intensity of tumour cells stained. These data were analysed in conjunction with clinical data.

### Results

Tissue from 75 patients was examined. Target expression scores varied widely between patients: NAT median 45%, inter-quartile range 25% - 65%; and SSTR2 median 55%, interquartile range 30% – 80%; and in some cases heterogeneity of expression between different parts of a tumour was observed. A weak positive correlation was observed between the expression scores of the different targets: correlation coefficient = 0.23,  $p = 0.05$ . *MYCN* amplified tumours had lower SSTR2 scores: mean difference 23% confidence interval 8% - 39%,  $p < 0.01$ . Survival did not differ by scores.

## **Conclusions**

As expression of both targets is variable and heterogeneous, imaging assessment of both may yield more clinical information than either alone. The clinical value of immunohistochemical assessment of target expression requires prospective evaluation.

Variable target expression within a patient may contribute to treatment failure.

## **Keywords**

Immunohistochemistry

Lutetium DOTATATE

meta-Iodobenzylguanidine

Molecular Radiotherapy

Neuroblastoma

Noradrenalin Transporter Molecule

Somatostatin Receptor

## Introduction

The childhood cancer neuroblastoma is stratified into low-, intermediate- and high-risk groups on the basis of the most powerful prognostic factors: stage, age and molecular pathology [1]. Despite sequential advances as a result of innovative multimodality treatment protocols [2-5], the prognosis of high-risk neuroblastoma remains poor, with the majority of patients dying from refractory or relapsed disease. Attempts to salvage these patients often focus on molecular radiotherapy with  $^{131}\text{I}$  meta-Iodobenzylguanidine (mIBG) [6, 7] or peptide receptor radionuclide therapy with, for example,  $^{177}\text{Lu}$  Lutetium DOTATATE [8], and a number of clinical trials investigating these treatments are in progress.

Molecular radiotherapy is regarded as a potentially important treatment option for high-risk neuroblastoma, because the disease is usually widely disseminated, has a poor prognosis, is known to be intrinsically radiosensitive, and, most importantly, expresses a range of specific molecular targets, which can be exploited therapeutically. These targets include: the noradrenaline transporter molecule (NAT); the somatostatin receptor (SSTR); and the diasialoganglioside GD2.

Noradrenaline is a catecholamine produced as a neurotransmitter and a hormone by sympathetic nerves and the adrenal medullary cells. NAT is encoded by the SLC6A2 gene [9], and is responsible for the active uptake of catecholamines and their analogues such as mIBG [10]. Most commonly labelled with  $^{123}\text{I}$ , mIBG has an established role in the staging and response assessment of neuroblastoma [11, 12]; and there is extensive experience in the

use of  $^{131}\text{I}$ -mIBG for the treatment of neuroblastoma [6]. While  $^{131}\text{I}$ -mIBG molecular radiotherapy has been clearly demonstrated to produce responses in a substantial proportion of patients with relapsed and refractory neuroblastoma, there have to date been no published randomised trials comparing this therapeutic approach with others. One randomised trial, however, is ongoing:  $^{131}\text{I}$ -mIBG alone versus  $^{131}\text{I}$ -mIBG with vincristine and irinotecan versus  $^{131}\text{I}$ -mIBG with vorinostat (New Approaches to Neuroblastoma Therapy Consortium Trial 2011-01, ClinicalTrials.gov identifier NCT02035137).

Somatostatin is a peptide hormone acting on SSTR in target tissues. There are five known SSTR subtypes, and SSTR2 is found within various normal tissues [13]. It is often highly expressed in neuroblastoma, and to a greater extent than other SSTR subtypes [14, 15]. Radiolabelled somatostatin analogues (for example  $^{111}\text{In}$  Indium Petetreotide [16] and  $^{68}\text{Ga}$  Gallium DOTATATE [8]) have been used for the imaging of neuroblastoma and other neuroendocrine cancers, and others (for example  $^{90}\text{Y}$  Yttrium DOTATOC [17] and  $^{177}\text{Lu}$  Lutetium DOTATATE [8]) have been used for therapy. A large randomised trial in patients with metastatic mid-gut neuroendocrine cancers has demonstrated that molecular radiotherapy with  $^{177}\text{Lu}$  Lutetium DOTATATE results in significantly better response rates, event free survival and mortality compared with a high-dose, long-acting release formulation of octreotide alone. A clinical trial in neuroblastoma is ongoing: A phase IIa trial of  $^{177}\text{Lu}$ -Lutetium DOTATATE in children with primary refractory or relapsed high-risk neuroblastoma (EudraCT Number: 2012-000510-10) [18].

Monoclonal antibodies directed against GD2 are an established treatment in the immunotherapy of neuroblastoma [4, 19], and radio-labelled versions have been explored as potential vectors for molecular radiotherapy [20].

Selection of patients with neuroblastoma for molecular radiotherapy with either  $^{131}\text{I}$ -mIBG or  $^{177}\text{Lu}$  DOTATATE depends on the use of nuclear medicine imaging as a predictive biomarker. We hypothesised that a better understanding of the tissue expression of molecular targets might help in patient selection for therapy, and allow optimisation of molecular radiotherapy in clinical practice. The recent commercial availability of monoclonal antibodies directed against SSTR2 and NAT for immunohistochemistry offered a way in which we could study target distribution in neuroblastoma tissue. The purpose of this study was to explore expression of NAT and SSTR2 in an unselected panel of archival tumour samples from patients for whom clinical prognostic factors and outcome were known, with the aim of using the findings to suggest potential ways in which molecular radiotherapy treatments might be improved.

## Materials and methods

Archived formalin fixed paraffin embedded (FFPE) neuroblastoma tissue samples, with associated patient clinical data including age, stage, *MYCN* status and survival outcome, were received from the Children's Cancer and Leukaemia Group (CCLG) tissue bank. Immunostaining was performed on a Leica Bond-Max machine. Commercially available antibodies for NAT and SSTR2 were used in this study. For NAT, a monoclonal antibody was obtained from Merck Millipore, Billerica, Massachusetts, USA (catalogue number MAB5620). For SSTR2, a monoclonal antibody, UMB1, produced by Epitomics, distributed by Insight Biotechnology, Wembley, UK (catalogue number 3582-1 AC-0162RUO) was used. The antibodies underwent in house checking on control organs known to contain tissues both positive and negative for antigen expression. This was adrenal gland for the NAT (medulla positive and cortex negative) and pancreas for the SSTR2 (islets positive and exocrine tissue negative).

The FFPE slides were baked at 60°C for 1 hour, then de-waxed and rehydrated through graded alcohol solution. A peroxidase block was then applied to the slide for 5 minutes. The primary antibody was then applied. For SSTR2 a dilution of 1:200 was applied for 15 minutes using heat induced epitope retrieval (HIER). For the NAT a dilution of 1:1000 was used post primary for 8 minutes. A polymer was applied for a further 8 minutes and diaminobenzidine for 10 minutes. Haematoxylin was applied for a further 5 minutes.



All slides were examined by two authors (JEG and NJS), blinded at the time to information on stage, age and MYCN status of the patient and blinded to the results of the other immunostaining.

The samples were categorised on morphology into differentiating, poorly differentiated and undifferentiated neuroblastomas according to standard criteria. Each sample was then semi-quantitatively scored for both of the antibodies (to NAT and SSTR2) for

1. Percentage cells staining with the antibody (0-100%)
2. The intensity of expression of the antibody (0-100%)

These scores were estimated to the nearest 10%. A composite score for the analysis (ranging from 0 to 100% in steps of 5%) was derived by adding the percentage cells staining with the percentage intensity of expression, and dividing by two [21, 22].

As the choice of cut-off points for categorising NAT and SSTR2 combined scores is not clear in neuroblastoma, and the prognostic influence of NAT and SSTR2 measures is also unclear, analysis of measures in their original continuous form was performed. This took values between 0 and 100% rather than grouping them into categories such as low, medium and high tertiles, because categorising a continuous measure results in loss of information and power. The results of immunohistochemical staining score for both NAT and SSTR2 were linked with known available data available from the CCLG tissue bank on age, stage, MYCN amplification and survival outcome.

Data were summarised using descriptive statistics and Pearson correlation was calculated for association between continuous measures. Regression and Cox proportional hazards models were used to compare groups (univariable and multivariable analyses) and calculate point estimates with corresponding 95% confidence intervals (CI) for the appropriate outcome measures respectively. Overall survival was calculated from the date of diagnosis to date of death, or censored at the date when the patient was last known to be alive for those patients whose death had not been reported. Functional form of independent variable was assessed using Martingale residuals. No adjustment for multiple testing was made. P-values below 0.05 were considered statistically significant. Stata v12 (StataCorp LLC, College Station, Texas, USA) was used for the calculations.

## Results

In total, 96 tissue samples from 88 patients were received from the CCLG tissue bank. Thirteen patients were excluded from the analysis – Wilms tumour (n=2), completely necrotic sample (n=1), normal tissue (n=4), ganglioneuroblastoma (n=4), no viable tumour (n=2). For five patients, two or more samples were sent, comprising in total eight additional samples. In these cases, the earliest tumour sample from the time of diagnosis was used for the statistical analysis. The cases with multiple samples are reported separately to see if receptor expression changed with treatment (See Table 1).

Tumour samples from 75 patients were available for statistical analysis. The majority were poorly differentiated neuroblastoma (n=62), with the others being characterised on morphology as differentiating (n=10) and undifferentiated (n=3).

Marked variation in expression of both targets in individual tumour samples was seen between patients for both the intensity and percentage expression of cells, and therefore the composite scores. Figure 1 illustrates the heterogeneity for SSTR2, where the observed values were: intensity of staining, median 60%, inter quartile range (IQR) 20% to 70%; proportion of cells staining, median 60%, IQR 10% to 90%; composite scores, median 55%, IQR 30% to 80%. Figure 2 illustrates the heterogeneity for NAT, where the observed values were: intensity of staining, median 30%, IQR 20% to 50%; proportion of cells staining, median 70%, IQR 20% to 80%; composite scores, median 45%, IQR 25% to 65%.

Expression of one target was weakly associated with expression of the other target. A weak correlation was observed between the expression of NAT and of SSTR2 composite scores, either when all patients were considered, or when only stage 4 patients were analysed separately. Figure 3 shows weak positive correlation between NAT and SSTR2 composite scores for all 75 patients (correlation coefficient = 0.23,  $p = 0.05$ ), and Figure 4 shows that there was a weak positive correlation between the receptor expression composite scores when just those 44 patients known to have stage 4 disease were examined (correlation coefficient = 0.14,  $p = 0.36$ ). An example of this variability of receptor expression is shown in Figure 5. This tumour expresses SSTR2 strongly, and NAT only weakly.

Within individual tumour samples, heterogeneity of target expression was sometimes observed as shown in Figures 6, 7 and 8.

Where multiple samples were received for the same patient, the expression of SSTR2 was observed to increase as the tumours changed with treatment from poorly differentiated to differentiated in all cases ( $n=5$ ) and in four out of the five cases for NAT. The data are shown in Table 1.

Summary statistics for SSTR2 and NAT expression composite scores are presented in Table 2 by each baseline characteristic. No significant association between patient gender, patient age or tumour stage and SSTR2 and NAT expression composite scores was noted.

Patients without MYCN amplification were noted to have significantly higher composite SSTR2 expression scores compared to patients with MYCN amplified with mean difference

of 23% (CI: 8% to 39%,  $p < 0.01$ ) and slightly higher composite NAT expression scores, mean difference 10% (CI: -3% to 23,  $p = 0.14$ ) than patients with MYCN amplification (Table 2).

Differentiating tumours were found to have higher composite SSTR2 expression scores with mean difference 30% (CI: 50% to 10%,  $p < 0.01$ ) and higher composite NAT expression scores, mean difference 30% (CI: 46% to 14%,  $p < 0.01$ ) than patients with poorly differentiated or undifferentiated tumours combined (Table 2).

Out of 75 patients, 26 deaths were reported. For five patients last follow up date was not available. Overall survival at 1 and 3 years were as follows: 86% (CI: 75% to 92%), and 67% (CI: 54% to 77%) respectively. There is no evidence to suggest that SSTR2 or NAT has a prognostic influence on overall survival with hazard ratio (HR) of 0.99 (CI: 0.98 to 1.01,  $p = 0.56$ ) and HR of 0.99 (CI: 0.97 to 1.01,  $p = 0.25$ ) respectively. The conclusions were unchanged when adjusting for important prognostic factors.

## Discussion

There have been a number of studies exploring the relationship between laboratory measurements of target expression, uptake of radiopharmaceuticals measured in the laboratory or by imaging, and clinical outcomes, with regard to both NAT and SSTR2 in neuroblastoma.

The majority of these have examined the mRNA levels rather than protein expression. Some have shown that neuroblastoma cell lines that lack the expression of NAT mRNA fail to accumulate mIBG [23-25], and in others the NAT mRNA level has correlated with the extent of mIBG uptake [25-27].

A recent study has examined whether there is an association between both the tumour NAT mRNA and the NAT protein expression with mIBG avidity in patients with neuroblastoma [21]. In contrast to the previous studies, no significant correlation between NAT mRNA expression and mIBG uptake was found: the median NAT mRNA expression level for 19 patients with mIBG avid tumours was 12.9% compared to 5.9% in the 8 patients with mIBG non-avid tumours ( $p=0.3$ ). However given the relatively small sample size, the lack of significant correlations should not be taken as evidence that there is no correlation, as this study only had the power to detect strong relationships. A significant correlation between NAT protein expression and mIBG avidity was however apparent: the median percentage expression was 50% in the mIBG avid patients compared to 10% in the mIBG non-avid patients ( $p=0.03$ ). Patients with mIBG avid tumours appeared to have higher NAT intensity

scores ( $p=0.06$ ). Only one out of the eight mIBG non-avid tumours had a NAT protein expression  $>2$ . As there were patients in the study who had low NAT protein expression but were still mIBG avid it was suggested that tumours with low NAT expression may accumulate mIBG via other transporters [21]. It seems therefore that protein expression (as we have assessed with immunohistochemical techniques) is more likely to be a valid biomarker than mRNA expression, although there have been no studies examining whether patients with greater NAT protein expression have a better response to  $^{131}\text{I}$ -mIBG molecular radiotherapy.

There has been one study, but with only five patients, which demonstrated a positive correlation between the protein expression of SSTR2 by IHC and mRNA of SSTR2 [28].

Previous studies have found that patients with high expression of SSTR2 mRNA had a better survival [28-30]. One study examined somatostatin concentrations (not SSTR2 mRNA or protein expression) by radioimmunoassay in 39 children,  $>12$  months of age and with stage 3 or 4 neuroblastoma and found that the 16 children with high somatostatin concentration had better survival than the 23 patients with low somatostatin concentrations ( $p=0.005$ ). No significant correlation was observed in 15 children between receptor expression as determined by  $^{111}\text{In}$ -pentetreotide scintigraphy for SSTR2 and somatostatin concentrations in tumour tissue [31].

A study of RT-PCR on 49 neuroblastoma samples found a wide-range of SSTR2 mRNA expression but it was lowest in stage 4 patients. The patients with greater SSTR2 mRNA expression had a better survival: approximately 90% five year overall survival compared

with 20% ( $p = 0.0005$ ). In nine patients a good correlation between the concentration of SSTR2 mRNA protein expression as demonstrated by uptake on  $^{111}\text{In}$ -pentetreotide imaging was found [32].

Several studies have shown that patients with MYCN amplification have lower expression of SSTR2 mRNA [29, 31], and a correlation between MYCN amplification and low NAT protein expression has been reported [21]. Within our cohort of patients, the patients with MYCN amplification had significantly lower composite scores for SSTR2, but the difference seen in the NAT score was not significant, possibly because of small sample size.

As discussed above, previous studies have used varying techniques to look for SSTR2 and NAT in neuroblastomas. None of the studies has correlated SSTR2 or NAT protein expression and survival. In terms of the relevance to functional imaging and molecular radiotherapy the protein expression is more relevant than the measurement of mRNA. There have been studies in adult neuroendocrine tumours that have found patients, with negative octreotide scans, but positive mRNA [33]. Studies in neuroblastoma have found mIBG uptake in patients with low levels of NAT mRNA [23]. It is also well recognized that there can be a poor correlation between mRNA and functional protein expression possibly due to post-translational modification.

For cases with multiple samples we found that the percentage of cells expressing NAT and SSTR2 increased as the tumours went became more differentiated, although the number of



cases with multiple samples was too few for an assessment of statistical significance. It is unclear from the published data whether the uptake of mIBG correlates with the differentiation of neuroblastoma cells. The *in vitro* data available has supported an increase in mIBG uptake after cellular differentiation [34, 35]. In contrast, the first reported *in vivo* studies, supported a correlation between higher mIBG uptake in the more undifferentiated neuroblastomas [36, 37]. However, this was not confirmed in an *in vivo* study, which concluded that it was impossible to make judgements about the differentiation of neuroblastomas depending on their mIBG uptake [38].

Our conclusions, based on our own observation of a high variation in both NAT and SSTR2 expression both within tumours and between patients, and the weak correlation between them, and taking into account other data reviewed here are, that as expression of the molecular targets at a cellular level is variable and heterogeneous:

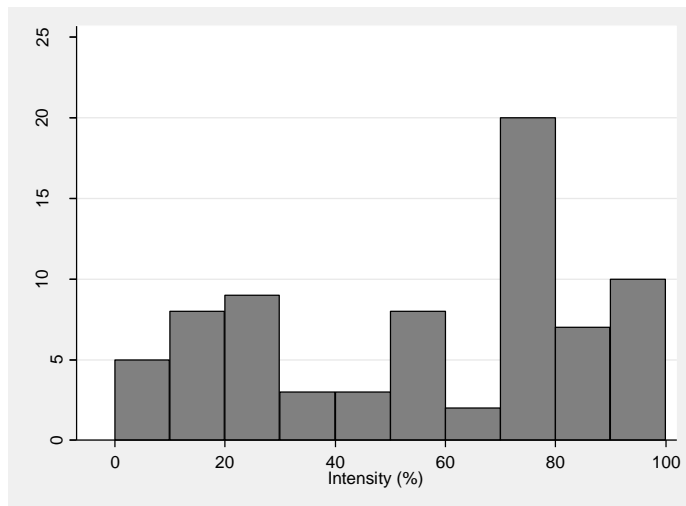
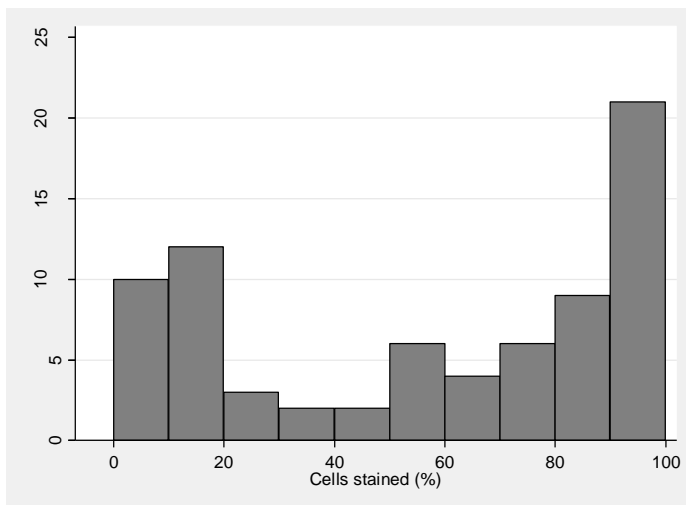
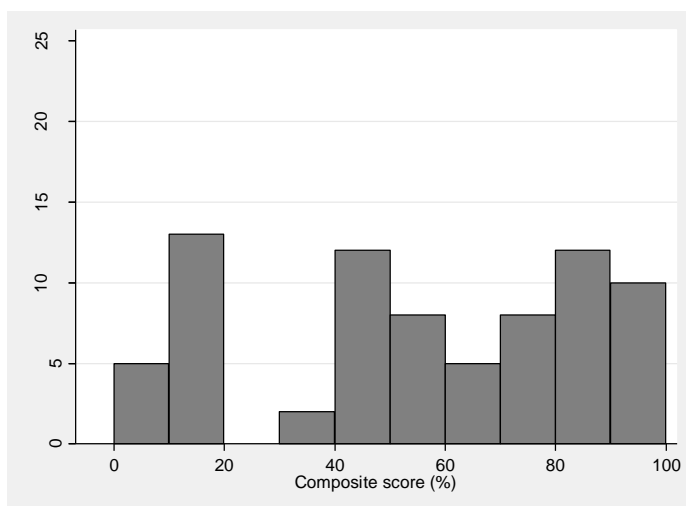
- 1 a more reliable clinical assessment of the extent of disease in individual patients may result from scans to demonstrate the expression of both NAT (e.g.  $^{123}\text{I}$ -mIBG scintigraphy and SPECT/CT) and SSTR2 (e.g.  $^{68}\text{Ga}$ -DOTATATE PET/CT), and
- 2 treatment schedules incorporating either NAT-directed (e.g.  $^{131}\text{I}$ -mIBG) or SSTR2-directed (e.g.  $^{177}\text{Lu}$ -DOTATATE) therapies may fail to target all disease.

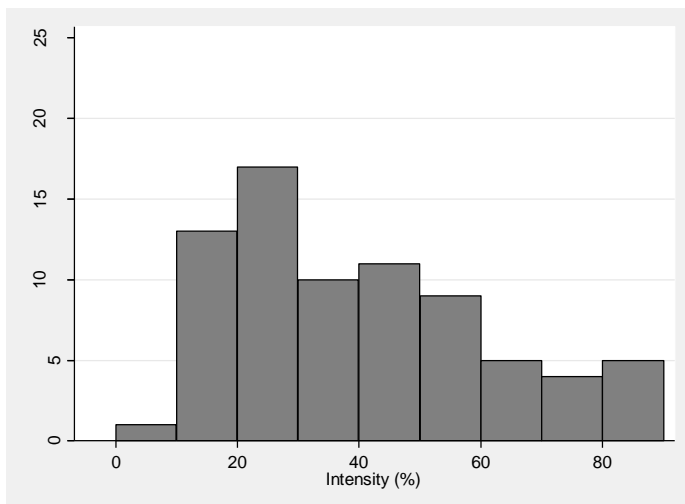
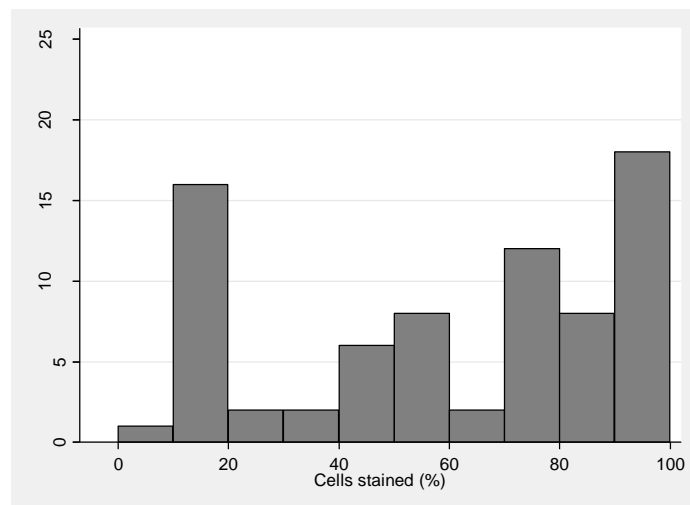
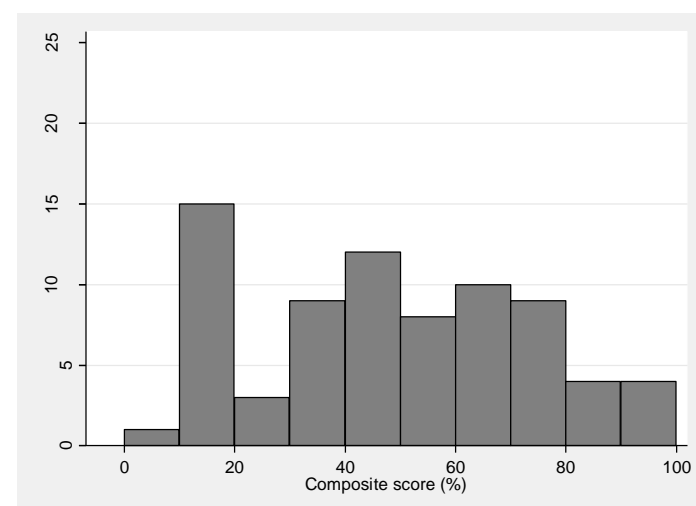
We hypothesise that the heterogeneity observed in target expression within individual patients may contribute to treatment failure in patients receiving molecular radiotherapy directed at a single target. We hypothesise that combined treatments directed at both NAT and SSTR2 may result in better coverage of tumour cells, with better clinical outcomes.

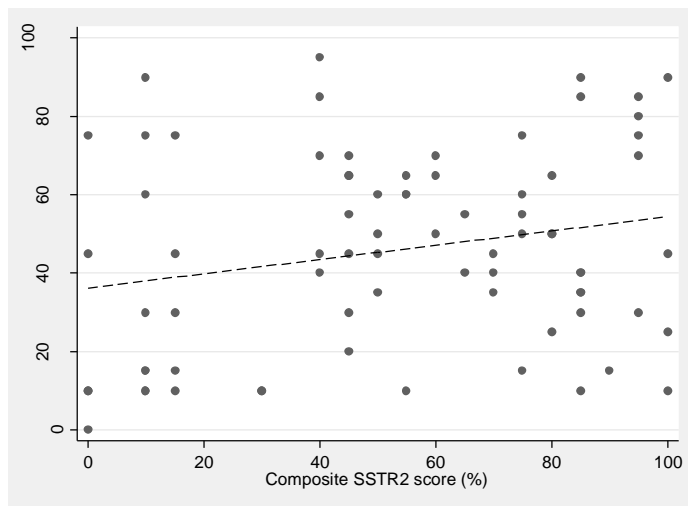
We recommend that future clinical trials of these therapies should have a prospective translational component assessing NAT and SSTR2 expression in biopsy samples, and correlate findings with imaging results and clinical outcomes. Laboratory studies and clinical trials should be designed to evaluate the hypothesis that clinical outcomes may be improved by combination treatments of NAT-directed and SSTR2-directed molecular radiotherapy.

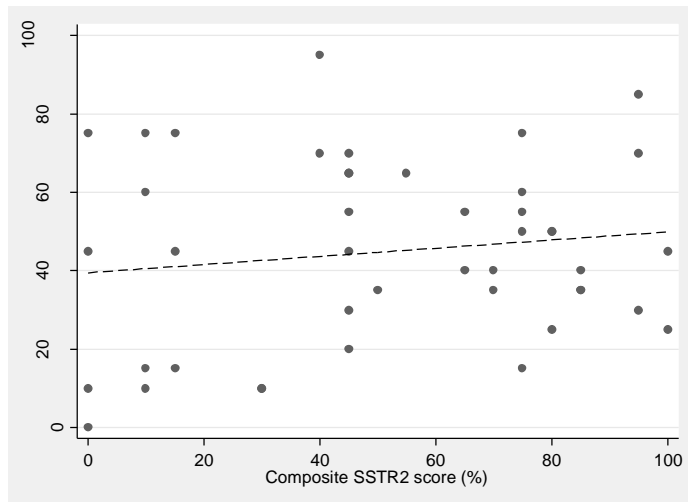
## Legends for Illustrations

- Figure 1     Histograms for SSTR2 expression in 75 patients showing distribution of (A) percentage intensity staining of cells, (B) percentage number of cells stained, and (C) composite scores.
- Figure 2     Histograms for NAT expression in 75 patients showing distribution of (A) percentage intensity staining of cells, (B) percentage number of cells stained, and (C) composite scores.
- Figure 3     Scatter plot of the composite scores for both SSTR2 and NAT in all 75 patients, showing weak correlation between the expression of the two receptor types.
- Figure 4     Scatter plot of the composite scores for both SSTR2 and NAT in stage 4 patients (n=44), showing weak correlation between the expression of the two receptor types.
- Figure 5     Photomicrograph of a tumour sample to show (A) intense staining with SSTR2 (100% of cells with 100% intensity) in contrast to (b) weak staining for NAT (B) (30% of cells with only 20% intensity) in the same part of the tumour.
- Figure 6     Example of variability of staining within a tumour sample: (A) H and E stain, (B) NAT staining and (C) SSTR2 staining. In this case the NAT and SSTR2 staining showed the same pattern of distribution.
- Figure 7     Further illustration of variable staining pattern within a tumour: (A) H and E stain, (B) SSTR2 staining and (C) NAT staining. There are areas of high and low expression of the SSTR2 and NAT corresponding to areas of well and poorly differentiated neuroblastoma on H and E.
- Figure 8     Example heterogeneity of target expression in relation to ganglion cell differentiation: (A) H and E stain, (B) SSTR2 staining and (C) NAT staining. This is more obviously seen on the SSTR2 staining than the NAT staining.

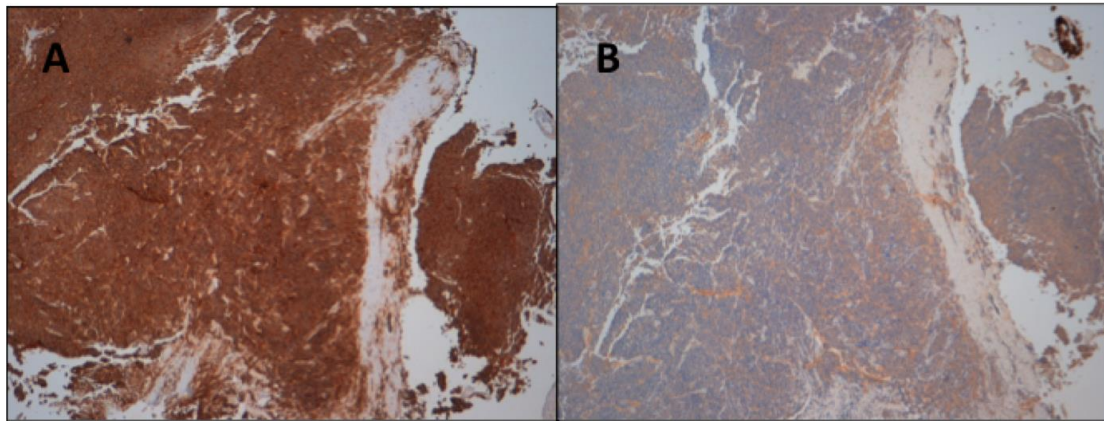
**Figure 1****A****B****C**

**Figure 2****A****B****C**

**Figure 3**

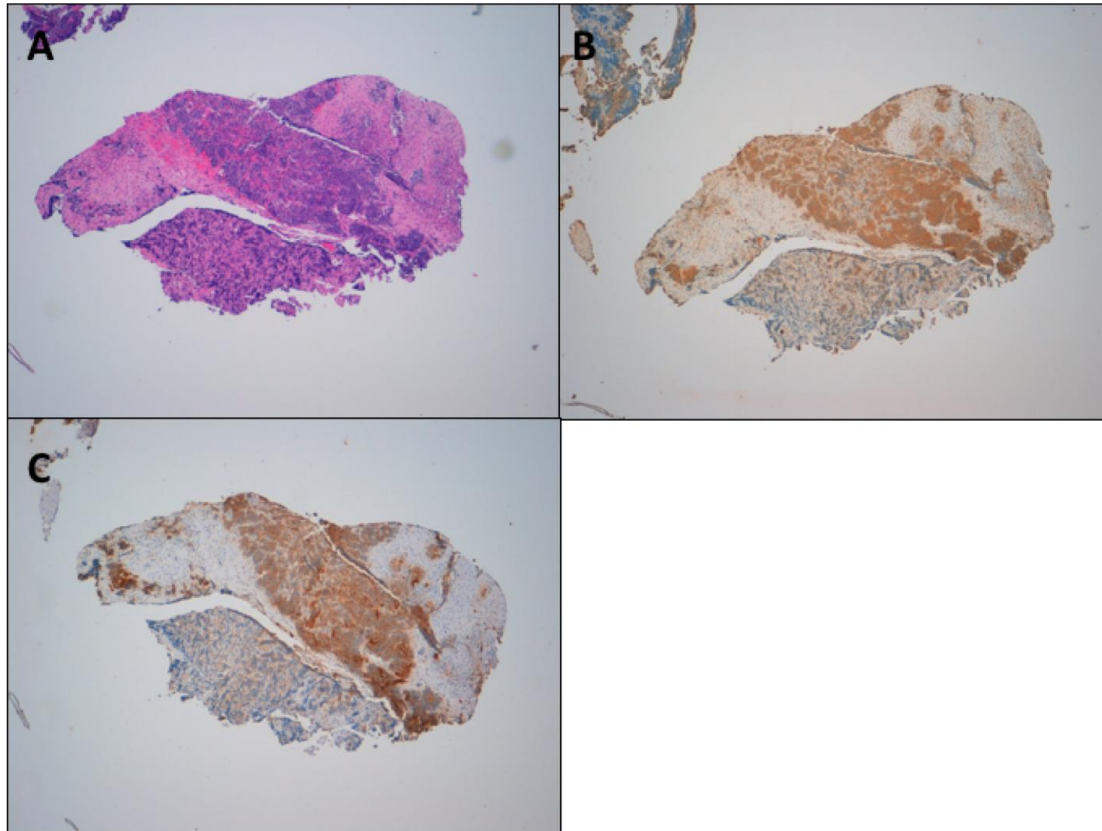
**Figure 4**

**Figure 5**

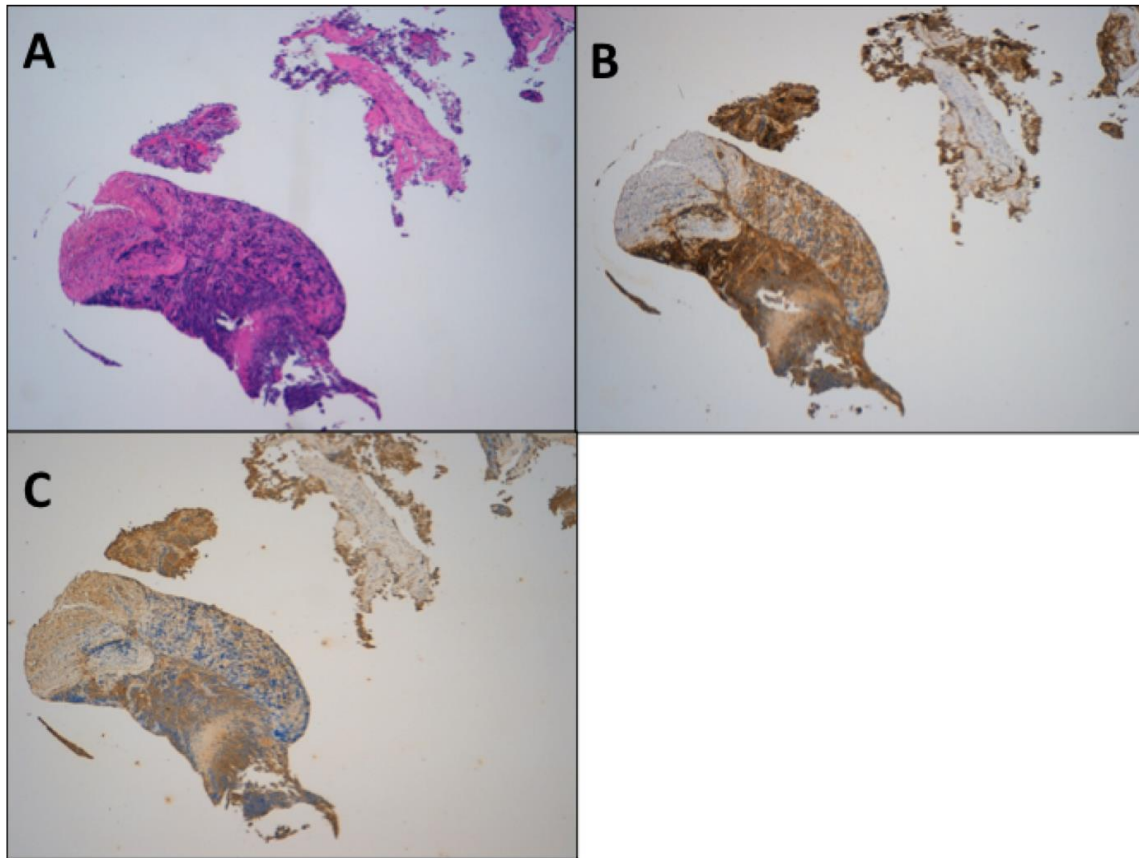




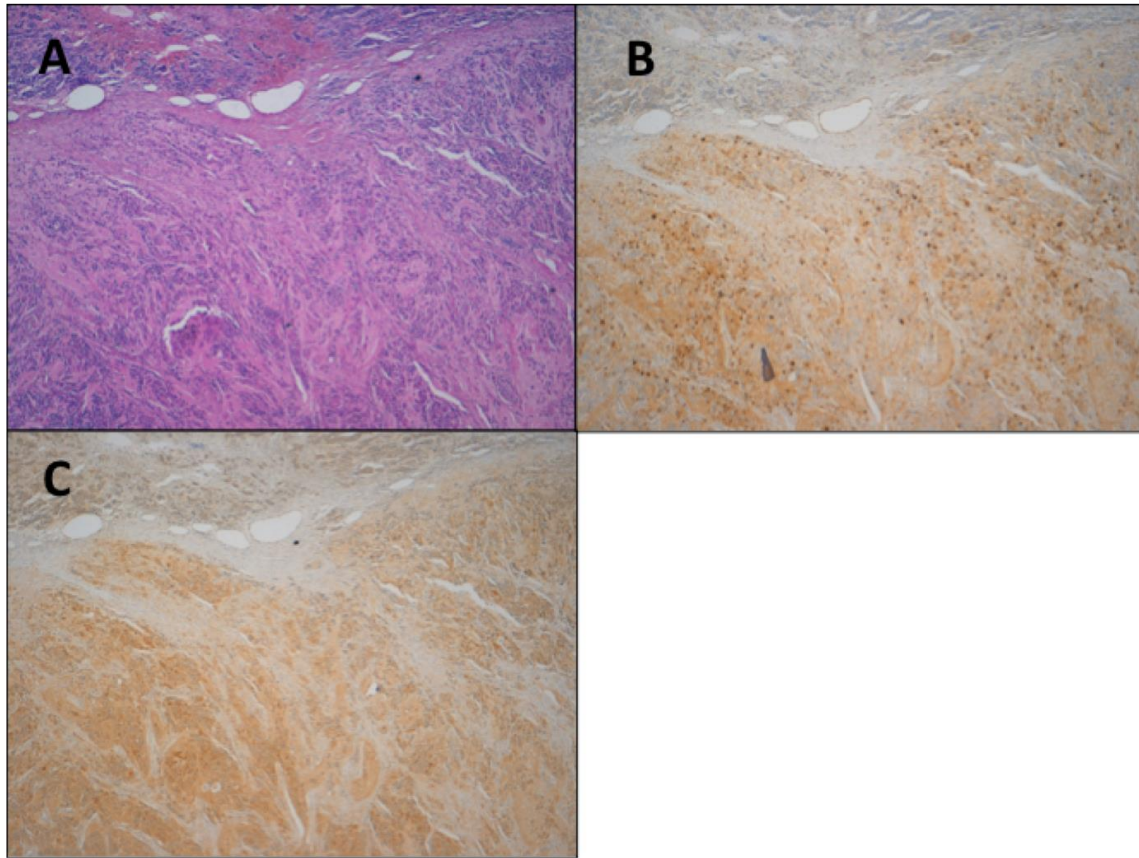
**Figure 6**



**Figure 7**



**Figure 8**



**Tables**

Table 1      The change in expression of NAT and SSTR2 with treatment in cases where multiple samples were sent.

Patient	Grade	SSTR2 Cells (%)	Intensity (%)	Composite score (%)	NAT Cells (%)	Intensity (%)	Composite scores (%)
1A	PD	70	70	70	40	30	35
1B	D	100	70	85	100	50	75
1C	D	100	70	85	100	50	75
2A	PD	80	90	85	10	10	10
2B	PD	40	30	35	20	20	20
2C	D	100	90	95	80	20	60
3A	PD	90	80	85	60	20	40
3B	D	100	70	85	80	30	55
4A	PD	50	50	50	30	30	30
4B	D	100	90	95	90	40	65
5A	PD	50	50	50	90	30	60
5B	D	100	90	95	30	40	35

Table 2 Summary statistics of SSTR2 and NAT combined scores by each baseline characteristic and patients' status.

Baseline characteristic		N	%	SSTR2 combined		NAT combined	
				scores (%)		scores (%)	
				(n=75)		(n=75)	
				Mean	SD	Mean	SD
Gender	Male	44	59	51	32	44	25
	Female	31	41	57	31	49	26
Age	18 month and under	35	47	53	32	47	28
	Over 18 month	39	52	55	31	45	23
Stage	I	3	4	73	20	53	28
	II	6	8	47	35	53	35
	III	6	8	75	22	42	27
	IV	44	59	52	32	45	23
	IVs	5	7	28	25	31	19
Differentiated	Differentiating	10	13	80	18	72	12
	Poorly differentiated	62	83	50	31	43	25
	Undifferentiated	3	4	30	44	27	18
MYCN	Yes	18	24	39	27	40	23
	No	48	64	62	29	50	25

**Compliance with Ethical Standards****Funding**

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**Conflict of interest**

All authors declare that they have no conflicts of interest as follows:

- Jennifer E. Gains declares that she has no conflict of interest
- Neil J. Sebire declares that he has no conflict of interest
- Veronica Moroz declares that she has no conflict of interest
- Keith Wheatley declares that he has no conflict of interest
- Mark N. Gaze declares that he has no conflict of interest

**Ethical approval**

This article does not contain any studies with animals performed by any of the authors.

This article does not contain any studies with human participants performed by any of the authors.

This article does contain a study on human tissue obtained from the Children's Cancer and Leukaemia Group Tissue Bank. All tissue banking procedures are performed in accordance with the ethical standards of the national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

**Informed consent**

Informed consent was obtained from all individual participants included in the study, or their parent or guardian, prior to tissue banking. This consent also prospectively covered subsequently approved studies on the tissue.

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