European Journal of Cancer (2014) **50**, 628–637

Available at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejcancer.com

Newly-derived neuroblastoma cell lines propagated in serum-free media recapitulate the genotype and phenotype of primary neuroblastoma tumours

Laurel T. Bate-Eya [a], Marli E. Ebus [a], Jan Koster [a], Ilona J.M. den Hartog [a], Danny A. Zwijnenburg [a], Linda Schild [a], Ida van der Ploeg [a], M. Emmy M. Dolman [a], Huib N. Caron [b], Rogier Versteeg [a], Jan J. Molenaar [a,*]

[a] *Department of Oncogenomics, Academic Medical Center, University of Amsterdam, Meibergdreef 15, PO Box 22700, 1105 AZ Amsterdam, The Netherlands*
[b] *Department of Paediatric Oncology, Academic Medical Center, University of Amsterdam, Meibergdreef 15, PO Box 22700, 1105 AZ Amsterdam, The Netherlands*

Available online 6 December 2013

KEYWORDS
Neuroblastoma
Neuroblastoma TICs
aCGH
Whole genome sequencing
Principal component analysis

Abstract Recently protocols have been devised for the culturing of cell lines from fresh tumours under serum-free conditions in defined neural stem cell medium. These cells, frequently called tumour initiating cells (TICs) closely retained characteristics of the tumours of origin. We report the isolation of eight newly-derived neuroblastoma TICs from six primary neuroblastoma tumours and two bone marrow metastases. The primary tumours from which these TICs were generated have previously been fully typed by whole genome sequencing (WGS). Array comparative genomic hybridisation (aCGH) analysis showed that TIC lines retained essential characteristics of the primary tumours and exhibited typical neuroblastoma chromosomal aberrations such as MYCN amplification, gain of chromosome 17q and deletion of 1p36. Protein analysis showed expression for neuroblastoma markers MYCN, NCAM, CHGA, DBH and TH while haematopoietic markers CD19 and CD11b were absent. We analysed the growth characteristics and confirmed tumour-forming potential using sphere-forming assays, subcutaneous and orthotopic injection of these cells into immune-compromised mice. Affymetrix mRNA expression profiling of TIC line xenografts showed an expression pattern more closely mimicking primary tumours compared to xenografts from classical cell lines. This establishes that these neuroblastoma TICs cultured under serum-free conditions are relevant and useful neuroblastoma tumour models.

* *Corresponding author:* Address: Department of Oncogenomics, M1-132, Academic Medical Center, University of Amsterdam, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands. Tel.: +31 205667536; fax: +31 6206918626.
E-mail address: j.j.molenaar@amc.uva.nl (J.J. Molenaar).

0959-8049/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.ejca.2013.11.015

1. Introduction

Neuroblastoma is a neuroendocrine tumour that arises from the peripheral sympathetic nervous system [1]. International Neuroblastoma Staging System (INSS) stage 1 and 2 tumours display excellent prognosis while stage 3 and 4 tumours have poor clinical outcome with a survival rate of 30% [2–5]. Neuroblastoma can genomically be characterised by aberrations such as gain of chromosome 17q, partial loss of chromosome 1p or 11q and MYCN amplification [6]. MYCN amplification occurs in about 20% of tumours and strongly correlates with poor prognosis [7,8]. Gain of 17q is the most frequent genomic abnormality which is present in over 90% of high grade neuroblastomas [9].

Cancer cell lines have been regarded as valid systems to study cancer biology. Nevertheless, these cell systems are cultured in non-physiological conditions and maintained for many years allowing considerable artificial adaptation. The genetic makeup and phenotypic characteristics of these cells can thus differ substantially from their original tumours [10,11]. In recent years, protocols have been devised to culture cells from fresh tumours in serum-free conditions in neural stem cell medium [12,13]. Such cells retained much of the characteristics of the original tumours and were able to initiate tumours in immune-compromised mice. They were therefore frequently called tumour initiating cells (TICs), a terminology that we use in this paper to refer to cells cultured by these protocols. TICs isolated from neuroblastoma have recently been reported, but the origin of these cells has been under debate [14–17].

Here we report the unambiguous establishment of TIC lines from neuroblastoma. These TICs reflect the primary tumours based on the genotype and phenotype and are not contaminated with cells from the haematopoietic lineage. We also show xenografts of neuroblastoma TICs recapitulate the genotype of primary neuroblastoma and showed similar mRNA expression compared to primary tumours as opposed to xenografts from classical neuroblastoma cell lines.

2. Materials and methods

2.1. Patients, isolation and culture of neuroblastoma TICs

Freshly resected human neuroblastoma tissue was obtained directly at surgery. Primary neuroblastoma cells were derived by mechanical disaggregation followed by enzymatic digestion of sheared tumour tissues with Liberase DH (500 μl/25 ml) (Roche) for 45 mins. Digests were passed through a 40 μM cell strainer (BD Biosciences) and cells were cultured in TIC total medium containing Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F12) (GIBCO) supplemented with 40 ng/ml basic fibroblast growth factor (bFGF), 20 ng/ml epidermal growth factor (EGF), 1× B27 supplement (GIBCO) and 500 U/ml of penicillin/streptomycin, 1000 mg/ml glucose and 110 mg/ml pyruvate. Neuroblastoma TICs were designated by 'AMC' (Academic Medical Centre), the name of the research institute, followed by the patient number and in case of TIC lines the letter 'T' or 'B' was added (T indicating that the TIC line was derived from the primary tumours and B from bone marrow metastases).

2.2. Array comparative genomic hybridisation (aCGH) analysis

aCGH was performed by hybridising 100 ng genomic DNA to a 180 K platform (Agilent Technologies). DNA was labelled by random priming with CY5-dCTP and CY3-dCTP respectively and hybridised at 65 °C for about 17 h. The chips were scanned on an Agilent G2565BA DNA microarray Agilent scanner. Digital analysis was performed with the R2 bioinformatics platform (http://r2.amc.nl).

2.3. mRNA expression profiling and principal component analysis

RNA was extracted from tumours and cell lines with TRIzol (Invitrogen, Carlsbad, CA) following the manufacturer's protocols. RNA concentration and quality were determined using the RNA 6000 Nano assay on the Agilent 2100 Bioanalyzer (Agilent Technologies). Fragmentation of cRNA, hybridisation to hg-u133 plus 2.0, microarrays and scanning were carried out according to the manufacturer's protocol (Affymetrix Inc., Santa Barbara, CA). The mRNA gene expression data were normalised with the MAS5.0 algorithm within the GCOS programme of Affymetrix Inc. Target intensity was set to 100. All data were analysed using the R2 genomic analysis and visualisation platform (http://r2.amc.nl).

2.4. Animal experimentation

Athymic NMRI-*nu/nu* and NOD-CB17-Prkdcscid/NCrHsd were subcutaneously injected with 1×10^6, 2.5×10^6 and 5×10^6 cells of the TIC lines. The size of the tumours was recorded for a period of about 3 months after which tumours were formalin fixed and paraffin sections routinely analysed by haematoxylin–eosin staining. Tumour pieces were serially xeno-transplanted into mice recipients for up to 14 passages. Orthotopic injection of luciferase 2 expressing AMC711T and AMC691B cells was also performed. Briefly, 1×10^6, 2.5×10^6 and 5×10^6 cells were injected into the fat tissue surrounding the adrenal glands of NMRI-*nu/nu*. Tumour take was evaluated after a period of 3 months by exponential increase of the intensity of luciferase signal within the mice using a highly cooled CCD camera.