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Detection Of Human *c-Myc* and *EGFR* Amplifications in Circulating Extracellular Vesicles in Mouse Tumour Models

Original Research Article

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Abstract Essentially, all cells release extracellular vesicles (EVs) that end up in biofluids, including blood, and the contents of these EVs can provide a window into the status of the cells from which they are released. This is particularly interesting in cancer, since these EVs allow for 'ex-vivo' analysis of the properties of the tumours without the need for biopsy. Gene mutations, rearrangements, amplifications, and epigenetic changes in the transcriptome can be monitored in circulating EVs. In this study, we used two human tumour cell lines derived from an epidermoid carcinoma and a medulloblastoma, which had amplification for the epidermal growth factor receptor *(EGFR)* and c-*Myc* genes, respectively. Cells were implanted subcutaneously into immunocompromised mice, and levels of gene amplification in both groups of subcutaneous tumours were quantified. We then determined if elevated levels of transcripts for the human *EGFR* and *c-Myc* were represented in circulating EVs in tumour-bearing mice. The expression levels of both human *EGFR* (*h-EGFR*) and human *c-Myc* (*h-c-Myc*) mRNAs in circulating EVs correlated well with their amplified status in the tumours. This data provides further support to the idea that circulating EVs are a potential platform for tumour biomarkers.

Keywords Cancer, Epidermoid Carcinoma, Extracellular Vesicles, Medulloblastoma, Biomarkers, Microvesicles, Gene Amplification

1. Introduction

Brain tumours comprise a variety of phenotypic and genotypic subtypes. Currently, genetic analysis is performed on biopsies obtained during surgical tumour resection or by surgical biopsy at a later date for suspected recurrences. As many newly developed drugs target specific pathways affected by the genetic status of the tumour [1], understanding specific transcriptome signatures that characterize them is critical for personalized clinical care.

Extracellular vesicles are nano-sized membrane bound particles released by essentially all cell types, including tumour cells, in vivo as well as in vitro, and comprise a mixed population of exosomes, shed microvesicles, and apoptotic blebs [2,3,4], collectively termed extracellular vesicles (EVs). They are found in all body fluids, including serum, plasma, cerebrospinal fluid (CSF), urine, and saliva [3], and can provide a window into the genotype and consequent phenotype of the tumour from which they are derived. The nucleic acid content of EVs provides a promising platform for companion diagnostics for monitoring tumour responses and changes during treatment [5, 6].

In the case of glioblastomas (GBMs), mutant oncogenic *EGFRvIII* mRNA and protein have been successfully detected in brain tumour-derived EVs found in the blood of GBM patients [6,7,8]. In addition, tumours are very dynamic entities and tend to change their genetic profile over time, especially after drug treatment and during recurrence. For example, lung cancer patients treated with *EGFR* tyrosine kinase inhibitors can relapse with a tumour harbouring a resistance mutation in *EGFR* [9]. This and other findings stress the importance of longitudinal tumour profiling to select and modify proper clinical treatment. Amplification of the epidermal growth factor receptor (*EGFR*) gene is a common event in GBMs and in about 40% of cases these amplified genomic fragments appear as double minute chromosomes [10]. Medulloblastoma is the most common and most malignant tumour of the central nervous system in children [11]. One histopathological feature that correlates with poor prognosis is large cell/anaplastic changes (20-25% of cases), which has been reported to be associated with amplifications in the c-*Myc* gene, with these amplifications being highly heterogeneous in the tumour tissue [11]. An EV-based assay for gene amplification would analyse nucleic acid derived from all cells in the tumour, thus having a better chance of representing different heterogeneous tumour characteristics.

In this study, the RNA content of circulating EVs released by human tumours in xenograft mouse models was examined. The following two cell lines were used: brain tumour medulloblastoma cell line amplified for c-*Myc*, but not *EGFR* [6], and a peripheral tumour epidermoid carcinoma cell line amplified for *EGFR*, but not *c-Myc* [12]. Tumour cells were injected subcutaneously into both flanks of immunocompromised mice and tumours allowed to grow for one month. RNA was extracted from serum-derived EVs, as well as from the subcutaneous (s.c.) tumour tissues. Genomic DNA (gDNA) was also extracted from the s.c. tumours. Levels of human *EGFR*, *c-Myc* and glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) mRNAs and gDNA were assessed by qRT-PCR and qPCR, respectively. Amplification of *EGFR* and *c-Myc* were found to be well reflected in the corresponding mRNA extracted from circulating EVs in mice bearing epidermoid carcinomas and medulloblastoma tumours, respectively. This data supports the idea that serum EVs can provide an important platform for cancer biomarkers, in this case tumour gene amplification, and can provide useful information to tailor treatment strategies.

2. Materials and Methods

2.1 Cell lines

Epidermoid carcinoma cell line A431 was obtained from ATCC and cultured in Dulbecco's modified essential medium (DMEM; Invitrogen, Carlsbad, CA USA) containing 10% foetal bovine serum (FBS; JRH Biosciences, Lenexa, KS) and penicillin/streptomycin (10 IU/ml and 10 μg/ml, respectively; Cellgro, Manassas, VA USA). Medulloblastoma cell lines D384 were obtained from Dr. Scott Pomeroy at Children's Hospital, Boston, MA and were originally generated at Duke University. Medulloblastoma cell lines were cultured in suspension in DMEM containing 10% FBS, 1×GlutaMAX (Invitrogen) and penicillin/streptomycin (as above).

2.2 Xenograft tumour models

A431 cells were cultured as monolayers, and on the day of injection they were rinsed in PBS twice, resuspended using trypsin, and then diluted in FBS-free DMEM and centrifuged at 300 x g for 5 min to pellet the cells. D384 cells were grown in suspension, and after being dispersed they were centrifuged for 5 min at 300 x g. The cell pellets (5.0×10^7 cells of each cell line) were resuspended in 500 μl of PBS and mixed with 500 μl Matrigel (BD Biosciences, San Jose, CA USA). Five million cells per flank were injected into each flank of all animals (total of 1.0×10^7 cells/mouse). Two groups of five adult mice (*nu/nu*, NCI) were each injected s.c. in both flanks with 100 μl/5×10^6 epidermoid carcinoma (A431; five mice) or medulloblastoma (D384; five mice) cells per injection. Tumours were allowed to grow for a maximum of one month. Blood was drawn by cardiac puncture while mice

were under deep anaesthesia prior to sacrifice. Approximately 1 ml blood was collected into Eppendorf tubes and allowed to clot at room temperature for 1 hr. Samples were then centrifuged at 1,300 x g for 10 min. Approximately 600 µl serum from 1 ml of blood was separated, filtered through a 0.8 µm filter and stored at -80°C until further processing. Tumours were also resected, weighed and snap frozen in liquid nitrogen. All animal procedures were performed according to the guidelines issued by the Committee of Animal Care of Massachusetts General Hospital.

2.3 Isolation of EVs and RNA extraction from serum and tumours

Serum samples were thawed on ice and centrifuged at 100,000 x g for 90 min using an Optima Max-XP, fixed angle MLA-55 rotor (*k* factor = 116; Beckman Coulter, Miami, FL, USA). The EVs pellets were lysed in Qiazol lysis buffer and RNA was extracted using the miRNeasy kit (Qiagen, Valencia, CA USA) according to the manufacturers' recommendations. Tumour tissue was homogenized and RNA was extracted using the miRNeasy kit (Qiagen). Then, 50 ng of RNA from tumour tissue or EVs was used for qRT-PCR. RNA yield and purity was assessed using NanoDrop 2000 (Thermo Scientific, Wilmington, DE USA).

2.4 Genomic DNA extraction

Around 20 µg of tumour tissue was cut from the frozen samples of each animal (epidermoid carcinomas and medulloblastomas) and lysed overnight at room temperature using the Blood and Tissue DNA extraction kit (Qiagen, Valencia CA) as suggested by the manufacturer's recommendations. The DNA was RNase treated using RNase A (Fermentas Inc., Glen Burnie, MD USA; 50 mg/ml) and the quality was assessed by NanoDrop 2000. Then, 10 ng of genomic DNA was used as input for each qPCR reaction.

2.5 Reverse transcription and qPCR

Twelve µL of the RNA from tumour tissue or EVs were reverse transcribed into cDNA using SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen), according to the manufacturer's recommendations. Samples were then preamplified using the TaqMan® PreAmp Master Mix (Applied Biosystems, Carlsbad, CA, USA). Briefly, 12.5µL of the cDNA was added to the PreAmp Master Mix together with human *EGFR*, *c-Myc*, and *GAPDH* primers and pre-amplified for 14 cycles, according to the manufacturer's recommendations. Primers for the preamplification step are the same as those used in the TaqMan PCR (see below). The samples were then diluted 1:10 and TaqMan qRT-PCR was performed on all samples for the corresponding cDNA. The amplification was performed using ABI PRISM 7500 with the following program: 50°C, 2 min; 95°C, 10 min; 40 cycles of 95°C, 15 s; 60°C, 1 min on standard mode. Primers and probes were as follows: *EGFR* Forward: TATGTCCTCATTGCCCTCAACA; *EGFR* Reverse: CTGATGATCTGCAGGTTTTCCA; Fam labelled probe AAGGAATTCGCTCCACTG; *c-Myc* Forward CAACCCTTGCCGCATCCAC ; c-*Myc* Reverse AGTCGCGTCCTTGCTCGG; Fam labelled probe: AGCAGCGGGCGGGCACTTTGCACT; *GAPDH* Applied Biosystems kit, catalogue number Hs03929097_g1. All assays were specific for the human genes and did not detect any mouse sequences.

2.6 Nanoparticle tracking analysis (NTA)

Serum samples were diluted 1:3000 in PBS and particles were counted using the NanoSight LM-10 system (NanoSight, Amesbury, UK) supplemented with a fast video capture and nanoparticle tracking analysis (NTA) software. Particles were recorded for 30 secs per run and analysed using the following settings: gain set at 10; detection threshold set between three and seven depending on sample concentration. Each sample was measured three times and the data expressed as mean ± standard deviation.

2.7 Statistics

Statistical analyses were performed using the Student's *t*-test (two tailed). A value of $p < 0.05$ was considered significant. All data is represented as mean ± standard deviation (SD).

3. Results

3.1 Primary tumour gene amplification is reflected in circulating EVs

Human epidermoid carcinoma (A431) and medulloblastoma (D384) tumour cells were injected subcutaneously into nude mice, and animals were monitored for one month. At the time of sacrifice, the mice were deeply anaesthetized and blood was collected by cardiac puncture, tumours were resected, and EVs were isolated from serum by high-speed ultracentrifugation for RNA qRT-PCR analysis. All data is normalized to *GAPDH* and represented as fold change compared to the tumour with the lowest levels of the gene of interest (i.e., non-amplified for that gene and has the lowest level within the group); A431 tumours are represented as fold change compared to the D384 tumour with the lowest levels of human *EGFR* (*h-EGFR*); and D384 tumours are represented as fold change compared to the A431 tumour with the lowest levels of human *c-Myc* (*h-c-Myc*). Human *EGFR* mRNA in tumour tissues from epidermoid

carcinoma (A431) xenografts was elevated between 1.2 x 10^5 and 3.5 x 10^5-fold compared to the medulloblastoma tumour D384 (not amplified for *EGFR*), while genomic DNA (gDNA) for h-*EGFR* was between 22 to 29-fold higher (Table 1). As expected, medulloblastoma xenograft tumour tissues (D384) had lower levels of h-*EGFR* mRNA in the range of 1.0-24-fold, with no elevation in h-*EGFR* gDNA levels (Table 1).

EVs isolated from the serum of mice injected with the epidermoid carcinoma cell line had elevated levels of h-*EGFR* mRNA in the range of 46—1.3 x 10^6-fold, while RNA from serum-EVs of medulloblastoma (D384) tumours had on average lower amounts of h-*EGFR* mRNA, ranging from 1.0-73-fold (Figure 1 and Table 1). Three animals injected with the medulloblastoma cell line (D384) had high levels of circulating h-*EGFR* RNA (31-73-fold), although the tumour tissue itself did not show any h-*EGFR* amplification, suggesting an increase in transcription rather than amplification (Table 1). On average there was about 100,000 times more h-*EGFR* mRNA in epidermoid carcinomas tumour tissue when compared to medulloblastoma tumour tissue ($p \leq 0.001$). The difference in h-*EGFR* mRNA levels in EVs from mice with epidermoid carcinoma tumours, as compared to medulloblastoma tumours, was also highly significant ($p \leq 0.001$; Figure 1A). EVs from the epidermoid carcinoma tumour in mouse A431–1 had the lowest levels of circulating h-*EGFR* RNA, which was more comparable to the levels of h-*EGFR* in three of the medulloblastoma injected animals.

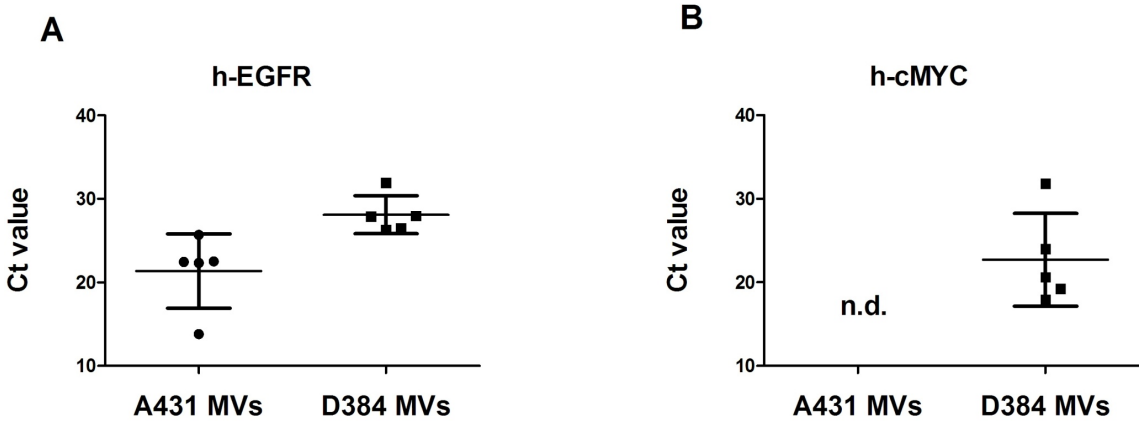

Figure 1. *EGFR* and *c-Myc* amplifications in the primary tumour are reflected in circulating EVs. Human *EGFR* and *c-Myc* mRNA levels were quantified in circulating EVs from xenograft models injected with either an epidermoid carcinoma cell line (A431; n=5) or a medulloblastoma cell line (D384; n=5), respectively. Box plot graph represents levels of h-*EGFR* (A) and h-*c-Myc* (B) mRNA in Ct values (lower Ct corresponds to higher levels of the message). The data is normalized to h-*GAPDH* mRNA levels. Values are represented as average ± S.D. (h-*EGFR* $p \leq 0.001$, as compared to D384 which is not amplified for *EGFR*; n=5 per group; n.d. = not detected).

	Tumour Tissue								Circulating EVs			
	h-*c-Myc*				h-*EGFR*				h-*c-Myc*		h-*EGFR*	
	gDNA	±*S.D.*	*mRNA fold*	±*S.D.*	*gDNA*	±*S.D.*	*mRNA fold*	±*S.D.*	*mRNA fold*	±*S.D.*	*mRNA fold*	±*S.D.*
A431 - 1	1.0	±0.8	2.2	±0.1	28	±1.1	257,905	±17,203	n.d.	n.d.	46	±0.4
A431 - 2	0.9	±0.1	1.0	±0.1	22	±1.3	122,516	±4,069	n.d.	n.d.	1,042	±148
A431 - 3	1.0	±0.2	2.5	±0.6	26	±1.4	219,609	±9,660	n.d.	n.d.	1,230	±89
A431 - 4	1.1	±0.1	3.7	±0.1	24	±1.0	354,072	±4,831	n.d.	n.d.	908	±26
A431 - 5	0.8	±0.1	3.9	±0.1	29	±1.3	154,010	±6,906	n.d.	n.d.	1,327,610	±64,262
D384 - 1	20	±1.2	45	±2.9	1.2	±0.1	1.1	±0.1	2,910	±356	73	±2.4
D384 - 2	21	±1.3	30	±0.6	1.2	±0.1	24	±0.3	1.962	±36	67	±0.4
D384 - 3	20	±1.1	39	±3.9	1.1	±0.2	1.4	±0.1	976	±30	31	±0.7
D384 – 4	19	±1.3	27	±1.1	1.0	±0.1	17	±0.2	126	±14	1.0	±0.1
D384 - 5	22	±1.1	51	±4.0	1.2	±0.2	1.0	±0.2	1.5	±0.6	2.1	±0.1

Table 1. Human *c-Myc* and *EGFR* mRNA fold change normalized to *GAPDH* in xenograft tumour tissue and circulating EVs as well as level of genomic DNA (gDNA)

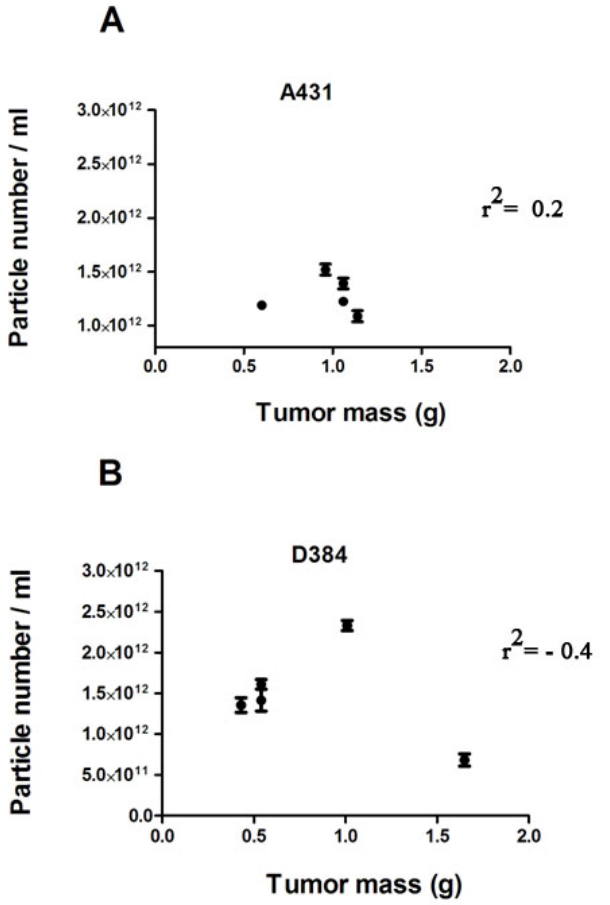

Figure 2. Particle counts and tumour sizes in mouse xenografts. Five animals were used in each group and injected in both flanks with either epidermoid carcinomas (A; A431) or medulloblastoma (B; D384) tumour cells. After one month tumours were resected, masses recorded and snap frozen in liquid nitrogen; serum was collected and stored at -80°C for gene amplification analysis. For NanoSight analysis, serum was diluted 1:3000 and particles were counted. Particle counts are represented as mean ± S.D. The Y-axis represents EVs counts and the X-axis represents tumour masses.

Human *c-Myc* mRNA in tumour tissues from medulloblastoma tumours (D384) was elevated between 27-51-fold compared to the *c-Myc* non-amplified epidermoid tumour (A431—1 set to 1), while gDNA for h-*c-Myc* was 19-22-fold higher (Table 1). The levels of circulating h-*c-Myc* at EVs reflected this amplification, ranging from a 1.5-2.910-fold increase as compared to the *c-Myc* non-amplified epidermoid carcinoma group (A431 set to Ct = 37/non-detectable). Human *c-Myc* RNA was not detected in EVs from any of the epidermoid carcinoma xenografts (Figure 1B and Table 1).

3.2 Xenograft tumour sizes and circulating EV (particle) counts

Medulloblastoma (D384) and epidermoid carcinoma (A431) tumours were resected after one month of in vivo growth. Tumours were weighed and recorded as follows: A431-1 – 1.14g, A431-2 – 0.96g, A431-3 – 1.06g, A431-4 – 1.06g, A431-5 – 0.6g, D384-1 – 1.01g, D384-2 – 0.43g, D384-3 – 0.54g, D384-4 – 0.54g, D384-5 – 1.65g. Epidermoid carcinoma (A431) and medulloblastoma (D384) tumour masses were on average 0.9 ± 0.2g (Figure 2A) and 0.8 ± 0.5g (Figure 2B), respectively. Serum samples from xenografts were diluted 1:3000 with PBS and counted using nanoparticle tracking analysis (NTA). Pearson correlation between particle number and tumour size revealed there to be no correlation in epidermoid carcinoma tumours (A431; r^2=0.2). Medulloblastoma tumours (D384) showed an initial correlation (4/5 mice) but the largest tumour had a low number of circulating EVs, rendering the correlation negative (r^2=-0.4; Figure 2B).

4. Discussion

Tumour-derived EVs provide a promising platform for cancer biomarkers. They provide a window into the genetic status of the tumour by allowing 'sampling' of the tumour from a distant, accessible site. Once released from tumour cells, EVs can end up in the bloodstream where they can be isolated and analysed for tumour markers and expression signatures. One of the major challenges of this field is the high background of EVs released from normal cells that contain a 'wild-type' transcriptome. Several approaches have been used to enrich for tumour-specific EVs and the field is currently expanding to include new and more advanced techniques, such as microfluidics [13], magnetic resonance imaging signals [8], antibody specific capturing [13], and streptavidin beads [14]. Currently, a widely used technique for EV isolation is ultracentrifugation, which sediments particles based on their size so that both normal and tumour cell-derived EVs are collected, although variations in recovery vary with fluid viscosities [15]. We have previously reported that human *c-Myc* RNA is found in circulating EVs from xenograft models injected with a medulloblastoma cell line [6]. In this study, we show that levels of gene amplification of *c-Myc* and *EGFR* in human tumour lines are reflected in circulating EVs. Xenograft mouse models were used and were injected with human cancer cell lines amplified for a specific oncogene, either *EGFR* (A431) or *c-Myc* (D384). Mice were then sacrificed and tumours removed and serum collected for RNA analysis of EVs. EVs were isolated by filtration and differential centrifugation, and RNA was analysed for quantity of human *EGFR*, *c-Myc* and *GAPDH* mRNAs.

Epidermoid carcinoma *EGFR* amplification was well-represented in circulating EVs from A431 mouse models. Overall, Ct values for *h-EGFR* mRNA in circulating EVs from mice bearing epidermoid carcinomas (A431) amplified at the genomic level for this oncogene were lower (corresponding to higher amounts; $p \leq 0.001$), than *h-EGFR* levels from medulloblastoma (D384)-derived EVs not amplified for this oncogene (Figure 1A). Human

EGFR gene amplification levels in the primary tumours were in concordance with what has been previously reported for cultured A431 cells [16]. Human *EGFR* mRNA levels in circulating EVs varied greatly among different tumour-bearing mice with one of them having 1.6 x 10^6-fold more *EGFR* message (compared to *GAPDH* message). This mouse did not have the highest levels of *EGFR* mRNA in the primary tumour or the highest number of circulating EVs (Figure 2A). In fact, this mouse had the smallest tumour size (0.6 g) in that group, but it appeared to be the most malignant tumour, as the mouse was cachexic (data not shown). This difference in the levels of *EGFR* mRNA in the primary tumour and circulating EVs may be due to increased transcription rates of mRNA and the subsequent more aggressive behaviour of the tumour. In addition, different copies of mRNA could exist at different locations, stabilities, and translations, and thus this kind of variation might be expected. High vascularization and invasion of the tumour may be another reason for such high malignancy, which in turn results in a lower body mass. Furthermore, only a small portion of circulating EVs are thought to be of tumour origin (5-10%), and this may indicate that these EVs exerted their immune-suppressive properties on the host immune cells, thus decreasing the total number of circulating normal EVs. This inhibition may have led to enrichment of the tumour-derived EVs and thus explains the high levels of h-*EGFR* mRNA in EVs detected by qPCR. Another epidermoid carcinoma in one mouse (A431-1) had very low levels of h-*EGFR* mRNA in circulating EVs (Table 1). The size of the tumour was similar to those in A431-2, A431-3 and A431-4 mice, but the number of vesicles was around 70% less compared to these three animals, which may account for the lower level of h-*EGFR* mRNA in circulating EVs (Figure 2).

Human *c-Myc* mRNA was detected in circulating EVs from all five xenografts injected with the medulloblastoma cell line (D384) and was not detected in circulating EVs isolated from mice injected with the epidermoid carcinoma (A431) cell line. Worth noting is that human *c-Myc* mRNA was not as high in the medulloblastoma tumour as *EGFR* mRNA was in the epidermoid carcinoma tumour (average mRNA increase was 29 vs. 1.1 x 10^5, respectively). The data suggests that, most likely, at least an 18-fold amplification for h-*c-Myc* in the tumour is required for detection in circulating EVs, while h-*EGFR* can be detected even when not amplified (data normalized to *GAPDH* and expressed as fold difference compared to h-*EGFR* non-amplified tumour [17]). One reason for this could be the difference in the half-life for these mRNA species, which is relatively short for *c-Myc* and therefore may be degraded before packaging inside EVs [18], or different localization of h-*c-Myc* and h-*EGFR* mRNAs inside the cells. According to the proposed kinetic model of RNA, genes encoding mRNAs with short half-lives are more likely to respond quickly to transcriptional activation or suppression [19]. Thus, the intrinsic stability of the mRNA would be expected to affect the rate of induction as well as the suppression of mRNA levels, because transcripts with short half-lives can respond more rapidly than stable transcripts to changes in transcription rates. The difference in h-*c-Myc* and h-*EGFR* RNA detection in EVs may also suggest specific packaging mechanisms for human *EGFR* mRNA into EVs, possibly exacerbated by the fact that this is occurring in a mouse background—especially because in 4/5 mice, h-*c-Myc* mRNA levels in EVs were higher than in the tumour itself. There seems to be a slight increase in EV numbers as tumour size increases, but this is not reflected in all animals. This may be due to the small sample size as well as the malignant nature of the tumour; in fact, epidermoid carcinomas (A431) cannot grow very large because the mouse quickly becomes heavily sick (data not shown), which may not allow enough time to detect an increase in circulating EVs. Medulloblastoma tumours, on the other hand, showed an initial increase in circulating EV number, but the animal with the largest tumour had a low circulating EVs count (Figure 2B), suggesting a higher malignancy, which may have inhibited the number of normal host-derived circulating EVs. Another variability might be the degree of vascularization of the tumour, which could lead to a different number of circulating EVs.

Within the limitations of an animal study, this study confirms the notion that EVs provide a potential platform for tumour biomarkers. Genetic mutations, rearrangements, and amplifications are reflected in RNA in EVs released by the tumour cells [20]. These EVs can be isolated from biofluids and provide a window into studying the primary tumour, either at diagnosis or later on for treatment response or follow-up studies.

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This work was performed in compliance with ethical research standards

6. Conflict of Interest

Johan Skog is an employee of Exosome Diagnostics. Thomas Wurdinger is an employee of ThromboDx. All other authors declare no conflicts of interest.

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