Identification of a novel inherited ALK variant M1199L in the WNT type of medulloblastoma

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Abstract

Rearrangements involving the ALK gene were identified in a variety of cancers, including paediatric tumour neuroblastoma where presence of ALK expression is also associated with adverse prognosis. Microarrays data indicate that ALK is expressed in another paediatric tumour – medulloblastoma. Therefore, we investigated if the ALK gene is mutated in medulloblastoma and performed simultaneously the molecular profiling of tumours.

Tumours from sixty-four medulloblastoma patients were studied for detection of ALK alterations in exons 23 and 25 using Sanger method. The molecular subtypes of tumours were identified by detection of mutations in the CTNNB1 gene, monosomy 6 and by immunohistochemistry using a panel of representative antibodies.

Among three ALK variants detected two resulted in intron variants (rs3738867, rs113866835) and the third one was a novel heterozygous variant c.3595A>T in exon 23 identified in the WNT type of tumour. It resulted in methionine to leucine substitution at codon position 1199 (M1199L) of the kinase domain of ALK protein. Results of analysis using three in silico algorithms confirmed the pathogenicity of this single nucleotide variation. The same gene alteration was detected in both patient and maternal peripheral blood leukocytes indicating an inherited type of the detected variant. Presence of ALK expression in tumour tissue was confirmed by immunohistochemistry. The tumour was diagnosed as classic medulloblastoma, however with visible areas of focal anaplastic features. The patient has been disease free for 6 years since diagnosis.

This is the first evidence of an inherited ALK variant in the WNT type of medulloblastoma, what altogether with presence of ALK expression may point towards involvement of the ALK gene in this type of tumours.

Key words: medulloblastoma, ALK gene, WNT type.

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Introduction

The ALK gene encodes a tyrosine kinase receptor which is expressed in the developing nervous systems [13]. The ALK gene is also expressed in neuroblastoma, the most common extracranial childhood tumour originating in the sympathetic nervous system and presence of expression is associated with adverse prognosis [8,26]. Importantly, ALK gene defects were reported both in terms of copy number changes and presence of somatic mutations in up to 10% of tumours, with two hotspot mutations in exon 23 (F1174L) and in exon 25 (R1275Q) [1]. Also germline ALK mutations were identified in both sporadic and familial cases of neuroblastoma [16,21] which altogether points to ALK as a key oncogene associated with this disease. Distribution of ALK mutations may be not random but typical for a particular molecular subtype of tumour. For example, F1174L substitution occurs more frequently in tumours with MYCN amplification [6].

Medulloblastoma, on the other hand, is the most common malignant intracranial childhood tumour of neuronal origin. Recent molecular studies revealed the existence of at least four molecular subtypes which display distinctive profiles of gene expression: Wingless (WNT), Sonic Hedgehog (SHH), Group 3 and Group 4 tumours [3,18,22]. Microarrays data indicate that ALK expression is associated with the WNT group, since the ALK gene was identified among top 50 most highly expressed genes in WNT tumours as compared to all other groups [18, in supplementary data]. Recent next generation sequencing studies in medulloblastoma based on analyses of ‘discovery sets’ of 37-92 tumours did not reveal any ALK gene alterations [17,23,24]. However the WNT groups analysed in all of these studies were small (from 5 to 7 tumours), thus preventing from discovery of changes occurring at low frequency. In support of that, the first ALK germline variant (3605delG) was discovered recently in a medulloblastoma patient pointing to a possible role of this gene in medulloblastoma [5].

Therefore, we further analysed exons 23 and 25 of the ALK gene together with molecular characteristics of tumours and found the second novel inherited ALK likely pathogenic variant in the WNT subtype of medulloblastoma.

Material and methods

Patients and tumour material

Sixty-four medulloblastoma patients treated from 1999 to 2014 in the Children’s Memorial Health Institute (CMHI) in Warsaw, Poland were included in the analysis.

Analysis was performed on frozen and formalin-fixed paraffin embedded (FFPE) tumours obtained at diagnosis. Hematoxylin-eosin-stained slides were analysed according to the current WHO 2007 criteria [20]. Large cell/anaplastic tumours (LCA) were diagnosed where anaplastic features were identified in the majority of analysed areas.

Additionally, genomic DNA extracted from the patient with ALK M1199L alteration and his parents’ peripheral blood leukocytes was used to confirm the germline character and parental origin of identified alteration.

Informed consent was obtained to use tumour and blood material according to the procedures outlined by the CMHI’s Ethical Committee.

Detection of molecular subtypes of tumours

The molecular subtypes of tumours were identified as follows:

1. WNT tumours by presence of at least two features as recommended by the International Medulloblastoma Working Group [12]: CTNNB1 mutation, immunohistochemical positive nuclear reaction against β-catenin (BD #610154, San Jose, USA, dilution 1:800) and presence of chromosome 6 monosomy detected either by interface fluorescence in situ hybridisation (FISH) or by multiplex ligation-dependent probe amplification (MLPA).

2. SHH tumours by presence of immunohistochemical positive reaction with anti-GAB1 (Abcam, Cambridge, USA, #ab27439 and/or ab #59362, dilution 1:800) and anti-YAP1 (Santa Cruz Biotechnology, Dallas, USA, #sc-101199, dilution 1:50) antibodies, as described by Ellison et al. [9].

3. Non-WNT/SHH tumours were the remaining tumours, tested negative for the above features.

Mutations in exon 3 of the CTNNB1

Mutations in exon 3 of the CTNNB1 gene were detected in genomic DNA obtained from frozen tumour tissues using the Sanger direct method. The
PCR reactions were carried out with the following primers: CTNNB1_3F:CCCTGGCTATCATTCTGCTT and CTNNB1_3R:TCTCTTTTCTTCCACCAAACTTT using AmpliTaq Gold DNA Polymerase (Roche, Basel, Switzerland) under the following conditions: 95°C for 8 min; 35 cycles of 95°C for 1 min; 57°C for 5 min; 72°C for 1 min, then a final extension step at 72°C for 7 min. Sequencing reactions were performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies) according to the manufacturer’s protocol. Sequencing products were analyzed in ABI Prism 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Sequences of the analyzed fragments were compared with the CTNNB1 cDNA (GenBank RefSeq: NM_001904.3) using Mutation Surveyor software version 3.30 (Soft Genetics, LLC, State Collage, PA, USA). The positions of the identified nucleotide changes were determined based on comparison with the reference sequence, with the A of the ATG translation initiation codon designated as nucleotide +1.

**Multiplex ligation-dependent probe amplification**

Multiplex ligation-dependent probe amplification was carried out on genomic DNA extracted from frozen tumour tissues for detection of copy number changes of chromosome 6. The analysis was performed using the SALSA MLPA kit P301-A2 (MRC-Holland, Amsterdam, the Netherlands) according to the manufacturer’s protocol. Probe amplification products were run an ABI Prism 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Peak plots were visualized and normalized, and the dosage ratios were calculated using GeneMarker software v 2.2.0 (Soft Genetics, LLC, State Collage, PA, USA).

**Fluorescence in situ hybridisation**

Fluorescence in situ hybridisation was performed on FFPE tissue preparations for detection of monosomy 6 using chromosome 6 Satellite Enumeration Probe (Kreatech, Amsterdam, the Netherlands) according to the protocols for the manufacturer of probes.

**Detection of ALK variants and gene expression**

A total of 20 ng of genomic DNA isolated from tumour tissue and/or peripheral blood lymphocytes by automated method (MagnaPure, Roche) was used to amplify coding and intronic flanking regions of exons 23 and 25 of the ALK gene.

The PCR reactions were carried out with the following primers: ALK_23F:GGAGCCTGCTGTGGTTCTTC and ALK_23R:AGTTGACACCCTGGGTTCC using AmpliTaq Gold DNA Polymerase (Roche, Basel, Switzerland) under the following conditions: 95°C for 8 min; 35 cycles at 95°C for 1 min; 62°C (exon 23)/60°C (exon 25) for 1 min; 72°C for 1 min, then a final extension step at 72°C for 7 min. Bidirectional sequencing was performed using a 3130 genetic analyser (Applied Biosystems, Foster City, CA, USA). The sequences were determined on both DNA strands from at least two independent PCR products. The analysed sequence fragments were compared with the ALK cDNA (GenBank RefSeq: NM_004304.4) and protein (GenBank RefSeq: NP_004295.2) sequences using Mutation Surveyor software version 3.30 (Soft Genetics, LLC, State Collage, PA, USA). Variant positions were numbered according to HGVS recommendations (with +1 corresponding to the A of the ATG translation initiation codon in the appropriate reference sequence).

Prediction of possible functional effects of novel alternations was performed using three different algorithms, which are web-based tools for the annotation of pathological variants in proteins: FATHMM (http://fathmm.biocompute.org.uk/index.html), PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), and Mutation Taster (http://www.mutationtaster.org/). The identified novel variants were also screened against mutation and SNP databases: NCBI (www.ncbi.nlm.nih.gov/SNP) and the Human Gene Mutation Database Professional (HGMD; www.hgmd.cf.ac.uk/ac/validate.php). Additionally, the amino acid position of all selected changes in functional domains and posttranslational modifications was verified using NCBI Protein (http://www.ncbi.nlm.nih.gov/protein) and Alamut-2.4-6 Software.

Expression of ALK was detected by immunohistochemistry using antibody clone ALK1 (DAKO #M 7195, Agilent Technologies, Santa Clara, USA, dilution 1 : 20).

**Results**

**Patients and tumours characteristics**

Tumours from sixty four paediatric patients with medulloblastoma were analysed. The average age of patients at diagnosis was nine years, range 0.5-17 years. Forty patients were males, 24 patients were females.
Molecular groups included: ten WNT tumours, eight SHH tumours, 41 non-WNT/SHH tumours and 5 tumours without determined molecular features due to lack of tumour material for extensive analyses. A summary of patients and tumour characteristics is presented in Table I.

**Detection of ALK variants**

Among sixty four tumours, five variants were detected in exons 23 and 25 presented in Table II. In four cases they resulted in an intron variant without impact on the function of the encoded protein and were not considered further.

In one tumour, a novel heterozygous likely pathogenic variant c.3595A>T was detected in exon 23 that resulted in a methionine to leucine substitution at codon position 1199 (M1199L) in the kinase domain of ALK protein (Fig. 1A). Analysis of genomic DNA obtained from proband’s peripheral blood leukocytes confirmed the germline nature of the

| Table I. Characteristics of 64 patients with medulloblastoma analysed for ALK variants |
|---------------------------------------------|-----------|-----------|-----------|-----------|
| No. of patients                            | ALL       | WNT       | SHH       | Non-WNT/SHH | Not determined |
| **Age (years)**                             |           |           |           |             |               |
| Average; range                             | 8.8; 0.5-17 | 9.3; 5-14 | 5.6; 0.5-11 | 9.2; 3-17 | 7.7; 2-15 |
| 0-3                                        | 5         | 0         | 4         | 0          | 1          |
| 4-17                                       | 59        | 10        | 4         | 41         | 4          |
| **Gender**                                 |           |           |           |             |               |
| Male                                       | 40        | 5         | 3         | 30         | 2          |
| Female                                     | 24        | 5         | 5         | 11         | 3          |
| **Histopathology**                         |           |           |           |             |               |
| Classic                                    | 44        | 10        | 1         | 32         | 1          |
| LCA¹                                       | 12        | 0         | 1         | 9          | 2          |
| DN²                                       | 5         | 0         | 5         | 0          | 0          |
| MBEN³                                      | 1         | 0         | 1         | 0          | 0          |
| Not available                              | 2         | –         | –         | –          | 2          |
| **ALK status**                             |           |           |           |             |               |
| Variant                                    | 5         | 2         | 0         | 2          | 1          |
| Normal                                     | 59        | 8         | 8         | 39         | 4          |

¹LCA – large cell/anaplastic, DN – desmoplastic/nodular, MBEN – medulloblastoma tumour with extensive nodularity

| Table II. Alterations in the ALK gene in five medulloblastoma tumours |
|---------------------------------------------|-----------|-----------|
| Group | ALK alterations¹ | Function |
| WNT   | c.3595A>T p. Met1199Leu | No change |
| WNT   | No change | c.3744-32A>G (rs3738867) | Intron variant |
| Non-WNT/SHH | No change | c.3744-32A>G (rs3738867) | Intron variant |
| Non-WNT/SHH | No change | c.3744-32A>G (rs3738867) | Intron variant |
| Not determined | c.3516-62A>G (rs113866835) | No change | Intron variant |

¹Alterations include intronic flanking regions of exons 23 and 25
detected variant. Also maternal DNA sample showed c.3595A>T alteration indicating an inherited type of the alteration.

Variant M1199L is located in the inhibitor binding region of the kinase domain with no recorded SNP site. To predict whether the identified variant is tolerated or deleterious, we used the combined results of three different algorithms (see methods), which confirmed the pathogenicity of the novel p.Met1199Leu variant in the ALK gene (FATHMM, PolyPhen-2, Mutation Taster Scores: -2.51, 0.7; 0.99; respectively). A novel variant position relative to two hot spots mutations found in neuroblastoma and the only mutation previously detected in medulloblastoma is presented in Figure 1B.

Characteristics of the patient and tumour with c.3595A>T ALK likely pathogenic variant

A ten-year-old boy was diagnosed in 2008 with brain tumour in the posterior fossa midline location. The tumour was surgically totally removed and no metastases were detected on magnetic resonance imaging examination. The patient is still alive and has been disease free for 6 years since diagnosis.

Histopathologically the tumour was diagnosed as classic medulloblastoma, however with clearly visible areas of focal anaplastic features (Fig. 2A-C). Analysis of DNA extracted from frozen tumour revealed presence of CTNNB1 mutation in exon 3 (c.98C>A; p.S33Y) and loss of whole chromosome 6 detected by MLPA analysis. In addition, positive nuclear reaction against β-catenin was identified by immunohistochemistry (Fig. 2D) what altogether classifies this tumour to the WNT group. Presence of ALK expression at the protein level was confirmed by immunohistochemistry using ALK1 antibody (Fig. 2E).

Discussion

Up to now around 30 recurrent chromosomal translocations involving the ALK gene have been identified in different cancers, mainly anaplastic large cell lymphoma (ALCL), non-small cell lung cancer (NSCLC), inflammatory myofibroblastic tumour (IMT) and other tumours (review in [25]). However, no fusion genes involving ALK have been found in neuroblastoma and recently described ALK translocations in neuroblastoma are not expected to result in a fusion transcript [10]. Therefore, mutations both somatic and germinal, leading to ALK activation are characteristic for this disease. It

Fig. 1. ALK molecular variants in medulloblastoma and neuroblastoma. A) A novel ALK likely pathogenic variant c.3595A>T (M1199L) in exon 23 in medulloblastoma. B) A “ribbon” rainbow diagram of human ALK protein. Red dots indicate location of mutations in medulloblastoma. The M1199L variant detected in this study is located in a hinge region of the kinase domain of the ALK protein. Purple dots indicate two hot spots mutations in neuroblastoma. F1174 is located in C-helix region and R1275Q in the activation segment of the kinase domain of the ALK protein. The view is prepared from protein data bank file RCSB PDB ID: 3L9P.
is likely that additional genetic changes (e.g. MYCN amplification) are required for neuroblastoma tumorigenesis. Mouse model experiments revealed that knock-in mice carrying Alk F1178L mutation (equivalent to human F1174L) were characterized by an increased number of sympathetic neuroblasts and a prolonged proliferation of sympathetic neurons but they did not develop neuroblastoma tumours [2].

We have analysed 64 medulloblastoma tumours, including ten of the WNT type, and found a novel ALK M1199L likely pathogenic variant in a binding region of the kinase domain, likely to be deleterious and inherited from maternal origin. This variant may be linked to the ALK inhibitors resistance since the contact maintained by methionine residue M-1199 is most important for the effective binding and stability of the ALK-crizotinib complex [19]. The neighbouring region includes also the sites of secondary acquired ALK alterations L1996M and L1998P in EML4-ALK fusion positive lung cancers which confer resistance to crizotinib, and G1202R, which confers resistance to a second-generation ALK inhibitor alec tinib and other ALK inhibitors [4,7,14,15]. Therefore, a precise character of the detected variant is critical for further therapeutic considerations.

Although the ALK gene was expressed at the protein level, it is possible that M1199L alteration is not sufficient for tumorigenesis alone and mutations in other genes are necessary for tumour development e.g. in CTNNB1 or as yet unknown genes from chromosome 6. On the other hand, mouse experiments uncovered that activating mutations in Ctnnb1 alone,
although caused the abnormal accumulation of cells on the embryonic dorsal brainstem, did not lead to the development of tumours [11]. This altogether emphasizes a requirement for a cooperative genetic event, similarly to neuroblastoma, in the development of the WNT type of medulloblastoma.

Germline deletion 3605delG found previously in medulloblastoma [5] is located within a short distance from the variant identified by us (c.3595A>T). However it is a nonsense mutation resulting in a frameshift producing a premature stop codon in exon 25 and putative truncated protein consisting of 1256 amino acids, as compared to 1620 of the wild-type. Its role in signal transduction remains to be determined. Also the molecular type of the tumour type. Its role in signal transduction remains to be determined. Also the molecular type of the tumour with ALK mutation was not established. Remarkably, both identified alterations occurred in tumours with presence of anaplastic features, which is in line with observation that ALK mutations are characteristic for de-differentiated tumours.

Very recent investigation of 37 medulloblastoma tumours did not reveal any mutations in exon 23 and exon 25 of the ALK gene [27]. It is likely that other alterations may be detected in medulloblastoma by application of e.g. next generation sequencing, similarly to intragenic ALK rearrangements detected recently in neuroblastoma [10].

In summary, this is the first evidence of inherited ALK likely pathogenic variant in the WNT type of medulloblastoma, what altogether with presence of ALK expression may point towards involvement of the ALK gene in this type of tumours.

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Disclosure

Authors report no conflict of interest.

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