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SHORT COMMUNICATION

Identification of *ALK* germline mutation (3605delG) in pediatric anaplastic medulloblastoma

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The *anaplastic lymphoma kinase* (*ALK*) gene has been found either rearranged or mutated in several neoplasms such as anaplastic large-cell lymphoma, non-small-cell lung cancer, neuroblastoma and anaplastic thyroid cancer. Medulloblastoma (MB) is an embryonic pediatric cancer arising from nervous system, a tissue in which *ALK* is expressed during embryonic development. We performed an *ALK* mutation screening in 52 MBs and we found a novel heterozygous germline deletion of a single base in exon 23 (3605delG) in a case with marked anaplasia. This G deletion results in a frameshift mutation producing a premature stop codon in exon 25 of *ALK* tyrosine kinase domain. We also screened three human MB cell lines without finding any mutation of *ALK* gene. Quantitative expression analysis of 16 out of 52 samples showed overexpression of *ALK* mRNA in three MBs. In the present study, we report the first mutation of *ALK* found in MB. Moreover, a deletion of *ALK* gene producing a stop codon has not been detected in human tumors up to now. Further investigations are now required to elucidate whether the truncated form of ALK may have a role in signal transduction.
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INTRODUCTION

Medulloblastoma (MB) is the commonest malignant brain tumor of childhood, arising from neuroectodermal embryonic tissues of the cerebellum. MB is classified into five variants: classic, nodular/desmoplastic, extensive nodularity, anaplastic and large-cell according to the World Health Organization.[1] These histological variants are heterogeneous with a variable clinical behavior. In particular, the anaplastic MB is typically associated with an aggressive clinical course.[2] MB cases have been reported in monozygotic and dizygotic twins[3,4] and may present in association with congenital cancer syndromes, including patients with germline mutations of *P53* (Li Fraumeni syndrome), *APC* (Turcot syndrome) and *PTCH1* (Gorlin syndrome).[5,6]

The *anaplastic lymphoma kinase* (*ALK*) is an orphan receptor tyrosine kinase (TK) often involved in genomic translocations in several human cancers, such as anaplastic large-cell lymphoma[7] and a subset of non-small-cell lung cancer.[8] Moreover, *ALK* germline and somatic missense mutations were discovered in familial and sporadic neuroblastoma,[9] and in anaplastic thyroid cancer.[10] In addition, we previously found that *ALK* overexpression is associated with advanced/metastatic neuroblastoma[11] and more recently it has been suggested that high levels of mutated and wild-type ALK mediate similar molecular functions that may contribute to a malignant phenotype in primary neuroblastoma.[12] As *ALK* is expressed during neuronal development[13] and has been found altered in several cancers, we performed a mutation screening of the *ALK* gene in pediatric MB in a cohort of 52 MB tumors and three human MB cell lines. Furthermore, *ALK* transcript expression was analyzed in a subset of 16 of these MBs.

MATERIALS AND METHODS

Patients, cell lines and nucleic acids isolation

Tumors were obtained from 52 patients with MB (39 classic, 6 nodular/desmoplastic, 1 extensive nodularity and 6 anaplastic) at the time of diagnosis, before any therapeutic regimen. Written informed consent was obtained from all patient parents or legal guardians. Tumors were classified according to the World Health Organization.[1] Genomic DNA was isolated from tumor samples and, when available, from peripheral blood lymphocytes, as well as from 135 healthy unrelated individuals using a standard phenol–chloroform protocol. Total RNA was extracted from 16 MB samples, using PerfectPure RNA Tissue Kit (5Prime, Hamburg, Germany). RNA quality was checked by 2100 BioAnalyzer using RNA 6000 Nano LabChip kit (Agilent Technologies, Santa Clara, CA, USA). DNA and RNA were also purified from the following human MB cell lines: DAOY, D341Med and D283Med (American Type Culture

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Collection, Manassas, VA, USA). All cell lines were cultured in Richter's Zinc Option medium, 10% fetal bovine serum and 1% non-essential amino acids.

ALK gene sequence analysis

A total of 30 ng of genomic DNA isolated from MB samples and peripheral blood lymphocytes, and MB cell lines were-PCR amplified for exons 20–28 of *ALK* and products were purified with ExoSAP-IT (GE-Healthcare, Waukesha, WI, USA). Bidirectional sequencing was performed by BigDye Terminator v1.1 kit (Life Technologies Corporation, Carlsbad, CA, USA) on the ABI-Prism 3130 genetic analyzer (Life Technologies Corporation). Sequencing outputs were analyzed with Sequencher version 4.8 software, (Gene Codes Corporation, Ann Arbor, MI, USA; http://www.genecodes.com).

ALK expression by quantitative PCR

RNA amplification and retro-transcription were performed by WT-Ovation RNA Amplification System (NuGEN Technologies, San Carlos, CA, USA). The relative expression of *ALK* was evaluated by quantitative PCR using a TaqMan Gene Expression Assay (Hs00608296_m1, Exons: 4–5; Life Technologies Corporation). We used *ATP5B, CYC1* and *EIF4A2* as reference genes as they were the most stable housekeeping genes from a set of tested candidate reference genes (geNorm; NuGEN Technologies). A gene expression normalization factor based on the geometric mean of the three housekeeping genes was calculated for each sample. Relative expression quantification was done with respect to a pool of nine RNAs extracted from cerebella tissues that were obtained from individuals, who died of causes unrelated to cancer and with an age ranging from 2 to 194 months. The lowest and highest values observed in the nine normal cerebellum samples were used as cutoffs for over- or underexpression of *ALK* in MB samples.

RESULTS

Mutation screening of *ALK* exons 20–28, which code for the entire TK domain of the receptor, on 52 MB tumors and 3 MB cell lines revealed a novel heterozygous deletion in exon 23 (3605delG) in one case of anaplastic subtype (Figure 1a). This mutation was also found in the constitutional DNA from the same patient and was confirmed by bidirectional sequencing in two independent PCR experiments. To exclude the possibility that the mutation was a polymorphism, we sequenced 135 healthy unrelated individuals and we did not detect such a mutation in any of them. This germline G deletion results in a

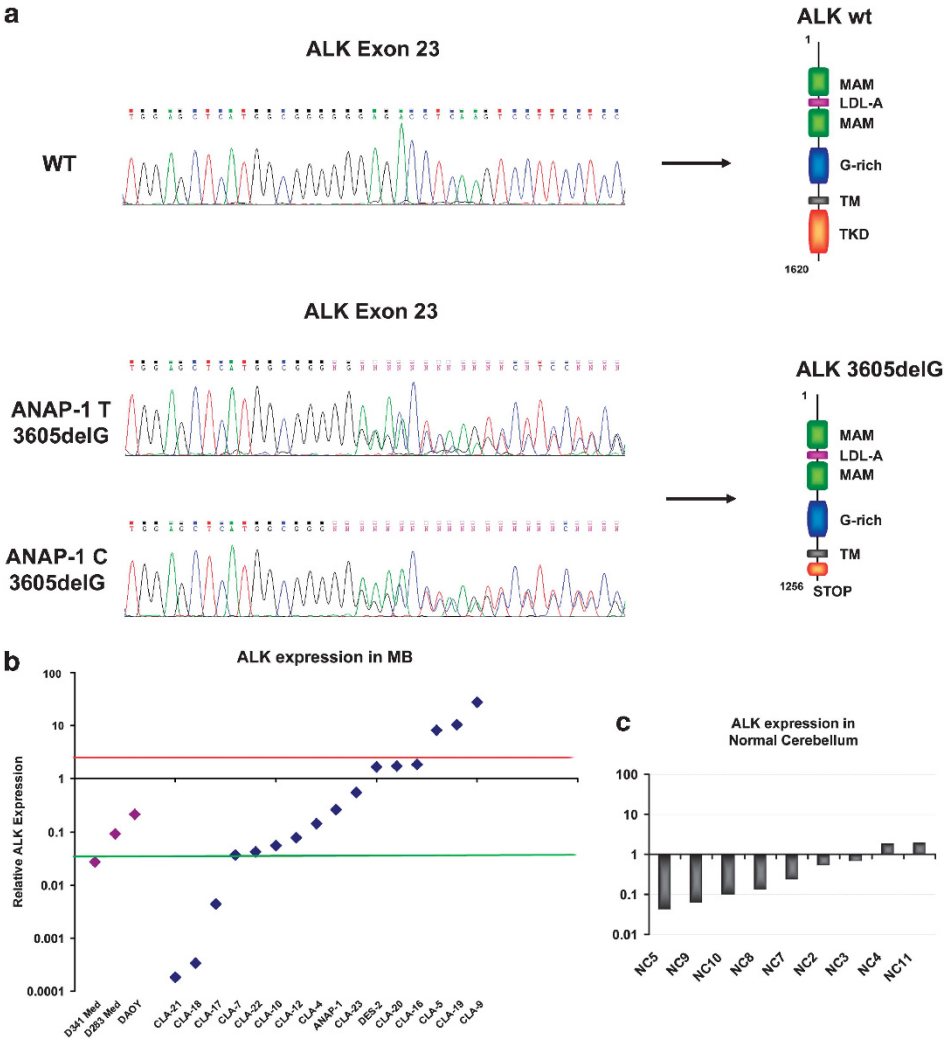

Figure 1 (**a**) Sequencing outputs of the genomic region within *ALK* exon 23 where 3605delG mutation was detected in the MB case ANAP-1. WT, wild-type; T, tumor; C, constitutional. The extracellular region of human ALK includes two MAM domains, one LDL-A domain and one glycine-rich domain. TM, transmembrane domain; TKD, tyrosine kinase domain. (**b**) Scatter plot chart of *ALK* relative expression values in 16 MB samples (blue) and 3 MB cell lines (purple). The relative expression of *ALK* was compared with a pool of normal cerebella obtained from nine unrelated individuals using the ΔC_t method. *ALK* downregulation cutoff was set for values $\leqslant 0.04$ (green line); *ALK* overexpression cutoff was set for values $\geqslant 1.98$ (red line). These cutoff values represent the highest and lowest value observed in normal cerebellum tissues. (**c**) Expression values of nine normal cerebella.

frameshift, thereby producing a premature stop codon in exon 25. The predicted putative protein would consist of 1256 amino acids against 1620 of the wild-type receptor (Figure 1a) and would lack most of the TK domain. This mutation was also detected at the cDNA level (data not shown). Nor *ALK* point mutations neither rearrangements were observed in the remaining MB samples and cell lines. We next investigated *ALK* mRNA expression in 16 MB tumor samples and 3 MB cell lines by quantitative PCR (Figure 1b). Relative gene expression analysis was performed with respect to a pool of RNAs extracted from nine normal cerebella, which yielded *ALK* expression values ranging from 0.04 to 1.98 (Figure 1c). The MB sample carrying the 3605delG *ALK* mutation had normal *ALK* mRNA expression (0.26), whereas *ALK* overexpression was detected in three classic MBs (Figure 1b).

DISCUSSION

Pediatric MB is an aggressive embryonic tumor of the cerebellum. In the present work, we screened a cohort of 52 MBs for mutations of *ALK*, a gene predominantly expressed in neural tissues during embryonic development. Here, we report the heterozygous germline mutation 3605delG in exon 23 of *ALK* gene in a MB sample with marked anaplasia, a histological variant with a significantly poorer prognosis than other MBs.[2] As far as we know there is no recurrence of MB in the family of this patient, but the lack of DNA samples from the parents prevented us to assess whether this deletion was inherited or a *de novo* mutation.

3605delG is the first nonsense mutation found in *ALK* gene and the only mutation reported in MB until now. This mutation is located within the TK domain, a region where *ALK* mutations have already been reported in both familial and sporadic neuroblastoma,[9] another embryonic tumor of nervous system. In neuroblastoma, *ALK* missense mutations lead to oncogenic activation of ALK through phosphorylation of the TK domain. More recently two novel gain-of-function mutations (C3592T and G3602A) have been discovered in exon 23 of *ALK* in anaplastic thyroid cancer.[10] Although 3605delG deletion was also detected at the cDNA level, and both wild-type and mutated alleles were transcribed, we did not have the possibility to analyze ALK protein expression and phosphorylation in this MB sample. Thus, we can just speculate that 3605delG mutation produces an impaired ALK protein, which would be truncated at amino acid 1256 and would lack most of the TK region. It has been shown for other TKs such as DDR1[14] that the kinase domain may not be necessary for covalent linkage, and that dimerization is likely mediated by the juxtamembrane, transmembrane and/or extracellular regions, which are intact in the 3605delG ALK mutant. Hence, the point of whether this deletion results in an activation of ALK remains unclear although it cannot be ruled out.

Although we analyzed a small cohort of patients, our results indicate that *ALK* mutations in MB should be rare, occurring in about 1–2% of cases. Nevertheless, in anaplastic large-cell lymphoma other *ALK* aberrations, such as inv(2)(p23; q35) ATIC-ALK, t(2;17)(p23; q23) CLTC-ALK and t(2; 19)(p23; p13.1) TPM4-ALK, occur at a similar low frequency.[15]

All above suggests that *ALK* gene is prone to mutations, which confer a high oncogenic potential in different cancers, although a confirmation of the oncogenic potential of 3605delG in MB is needed. Furthermore, as detected in this MB sample, most *ALK* mutations have been found in undifferentiated cancers, showing a histotype with anaplastic characteristics.[7,10] It is noteworthy that 3 out of 16 MBs showed overexpression of *ALK* mRNA. Overexpression of ALK has been proposed as an oncogenic mechanism in neuroblastoma, contributing to tumor aggressiveness,[11,12] but the point of whether ALK overexpression would be oncogenic in MB has to be further addressed. Quantitative expression analysis showed a normal *ALK* mRNA expression in the MB tumor with *ALK* mutation.

In conclusion, we report the first *ALK* mutation found in pediatric MB with anaplasia, showing that also in MB *ALK* is mutated although with a low frequency.

A screening of a larger number of cases, in particular belonging to the anaplastic variant of MBs, would be needed to better evaluate the role that *ALK* gene might have in the carcinogenesis and aggressiveness of MBs. Further investigations are now required to elucidate whether the truncated form of ALK may have a role in signal transduction.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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