Review of Potential Significance of Mutations of ADAMTS20, NF1 and PKHD1 Detected Using Next Generation Sequencing (NGS) in Dermal Fibrosarcoma Arising in Dermatofibrosarcoma Protuberans

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Abstract

We examined a status of fibrosarcoma arising in dermatofibrosarcoma protuberans of 64-year-old male patient. A dermal, solid, grayish-yellow, desmin-negative trichrome-bluish tumor measured 1.5 cm in diameter pT1a (eighth edition pTNM). It was composed of spindle cells. It was consistent with dermatofibrosarcoma protuberans (ICD-O3: 8832/3) in areas of low mitotic activity, low atypia and sustained CD34 positivity. CD34-negative texture with high mitotic index and atypia was consistent with the high grade sarcoma apparently of fibrous origin, given category of poorly differentiated fibrosarcoma. The high grade component was graded (G3) and scored according to French Federation of Cancer Centers Sarcoma Group (FNCLCC): total score of 6 points: tumor differentiation: 3 points + Mitotic count: 3 points (up to 26 mitoses/ 10HPF in high-grade fields), + no necrosis: 0 points. In low grade sarcomatous component ADAMTS20 (NM_025003: c.1661C>T, p.P554L) NF1 (NM_001042492: c. 2173G>T, p.E725X) and PKHD1 (NM_138694: c. 11074C>T, p.R3692X) were revealed with following allelic frequencies: 25%, 27% and 17%. In high grade component allelic frequencies of the same mentioned mutations were 30%, 30% and 14% respectively. In the light of our findings, none of detected mutations can be regarded as a mutation that would definitely induce phenotype of high malignancy, because ADAMTS20, NF1 and PKHD1 mutations were detected both in high grade sarcoma and in low grade areas of dermatofibrosarcoma protuberans. It also points that these mutations appeared on early stages of tumor development.

Keywords

ADAMTS20, dermatofibrosarcoma protuberans, fibrosarcoma, NF1 and PKHD1
INTRODUCTION

Dermatofibrosarcoma protuberas (DFSP) is a well-known low-grade soft tissue malignancy with local invasion and only exceptional metastases. At first its differential diagnosis evaluates from distinction from benign dermatofibroma. It was successfully based on appliance of immunohistochemical markers CD34 (being positive in DFSPs and usually negative in dermatofibromas (DFs) and factor XIIIa (giving inverse immunohistochemical results). However, it has always been a subject of concern due to its risk of progression to high grade sarcoma as fibrosarcoma or undifferentiated sarcoma. Therefore, to deepen our knowledge of this neoplasm a variety of research has been implemented to provide molecular landscape of genetic changes that characterize dermatofibrosarcoma protuberas. The defining abnormalities of this neoplasm on gene level as extra ring chromosome from 17q and 22q, or t(17;22)(q22;q13) could be absent in presence of an additional chromosome containing 8 or arm 8q as well as some segments of chromosomes 7, 17, 21, and 22 including two copies of a 17;22 fusion. Further, it was found that COLIA1-PDGFβ gene fusion is one of the molecular drivers of dermatofibrosarcoma growth. One of intriguing questions is which of genetic abnormalities are drivers of neoplastic progression of dermatofibrosarcoma to high grade sarcoma. In this search it was found that phosphorylation of unmuted EGFR and subsequent activation of its downstream factors mTOR and STAT5a/b play a role in dermatofibrosarcoma protuberas progression to high grade sarcoma. Similarly, the phosphorylation of Akt-mTOR pathway proteins (Akt, mTOR, 4EBP1, and S6RP) and PDGFRA/β was found to be present and correlated with each other on immunohistochemical level in fibrosarcomatous progression of dermatofibrosarcoma protuberas.

By way of further molecular explorations, we examined a status of potential hot spots in 409 tumor genes by means of next generation sequencing (NGS) in dermal high grade sarcoma arising in dermatofibrosarcoma protuberas of 64-year-old male patient. NGS was preformed separately for distinct textures of low grade sarcoma and high grade sarcoma.

MATERIALS AND METHODS

DNA isolation

In order to isolate DNA from formalin-fixed paraffin embedded tissue (FFPE), the commercially available Maxwell<sup>®</sup> 16 FFPE Plus LEV DNA Purification Kit (Promega, USA), dedicated to the Maxwell 16 MDx (Promega, USA), was used. The paraffin from the test samples was removed by subsequent rinsing using xylene (2-times) and ethyl alcohol (2-times). After deparaffinization, slides were dried at room temperature. The fragments of tissues (containing tumor cells) were transferred to Eppendorf tubes containing incubation buffer and proteinase K and incubated overnight at 70°C. Then samples were applied to cassettes containing a set of reagents enabling isolation. The next steps of the procedure were carried out in accordance with the manufacturer's instructions (Promega, USA). The DNA concentration was measured using a Qubit 2.0 fluorometer and the HS Assay Kit dsDNA (Thermo Fisher Scientific, USA) according to the manufacturer’s instructions. The average concentration for the obtained DNA isolates was 25 ng/ul, while the purity was 260/280 1.8-2.0.

Next Generation Sequencing

The isolated DNA was then used to detect mutations by the NGS method, using the Ion AmpliSeq™ Comprehensive Cancer Panel kit (Thermo Fisher Scientific, USA) that allows the study of 409 tumor genes. This panel includes tumor suppressor genes and oncogenes that are most often found mutated in tumor tissues and that: (i) are involved in tumor formation, (ii) are targets for therapy, (iii) are involved in signal transduction, (iv) apoptosis, repair DNA, regulation of transcription or (v) are responsible for inflammatory reactions.

Library preparation

The DNA was diluted to 10 ng/ul. The libraries were prepared using the Ion AmpliSeq™ Library Kit 2.0, the Ion AmpliSeq™ Comprehensive Cancer Panel kit and the Ion Xpress Barcode Adapters Kit (Thermo Fisher Scientific), according to the manufacturer’s instructions (Thermo Fisher Scientific). Comprehensive Cancer Panel, used for multiplex PCR reactions, contains 16,000 primer pairs, divided into four separate sets, 4000 pairs each. The average length of the amplified fragments is 155bp. Four separate PCR-multiplex reactions were performed for each of the samples tested, and the samples were combined into one. The resulting multiplex PCR products were subjected to partial enzymatic digestion to remove primer sequences. Next, adapters for multiplex PCR products were enzymatically attached using the Ion Xpress Barcode Adapters Kit (Thermo Fisher Scientific). One of the adapters contains barcodes that allow identification of sequences from a given patient among a mixture of libraries. The prepared libraries were cleaned using Agencourt AMPure XP (Beckman Coulter Genomics) according to the manufacturer’s instructions (Ion AmpliSeq Library Kit 2.0 - Thermo Fisher Scientific).

Preparation of clonally amplified template for sequencing - emulsion PCR (emPCR) for S5 using IonChef

The concentration of libraries was measured by quantitative PCR with real-time detection (qRT-PCR) using the
Ion Library TaqMan™ Quantitation Kit (Thermo Fisher Scientific) on a Rotor-Gene Q instrument (Qiagen). Based on the values obtained with qRT-PCR, all prepared libraries were diluted to a concentration of 100pM. Then, with Ion Chef (Thermo Fisher Scientific) and Ion 520 & Ion 530 Kit-Chef and Ion 530 ™ Chip Kit (Thermo Fisher Scientific), emPCR was performed, enrichment and two 530 chips were loaded (4 samples per chip).

**Sequencing**

Sequencing was performed on an Ion S5 sequencer (Thermo Fisher Scientific). Two 530 chips were used. Sequencing was done according to the manufacturer’s instructions.

**Bioinformatic analysis**

The raw data generated during sequencing was processed using the Torrent Server Suite 5.6-TSS (Thermo Scientific, USA). The obtained sequences were matched (mapped) to the reference sequence of the human genome (hg19). Searching for different variants (SNP, deletions, insertions) was carried out using the Variant Caller 5.6 program which is part of Torrent Server Suite 5.6. The following basic parameters of the variants were used: minimum allele frequency - SNP = 0.01 / INDEL = 0.05, minimal quality -10, minimal sequencing depth - 10. Variant Caller is compatible with the IGV genomic browser - Integrative Genomics Viewer (Broad Institute), which enables fast visualization of sophisticated variants. To annotate the detected variants with the TSS, the wANNOVAR software (www.wannovar.usc.edu) was applied. Additionally, Torrent Server Suite 5.6 generated FASTQ files that were used for analysis using the CLC Biomedical Workbench 5.0 (QIAGEN). The basic parameters used in the analysis were for CLC: minimum allele frequency - 0.01, minimal quality -10, minimal sequencing depth - 100. Detected mutations, SNP, insertions and deletions of the coding regions of the analyzed genes were filtered to detect pathogenic mutations by COSMIC base, dbSNP database (to discard hereditary polymorphisms) and population base of the 1000GENOMES project. The data analysis was also based on the internal clinical database of the Department of Molecular Diagnostics of Holycross Cancer Center. The Varsome program (https://varsome.com/) was also applied to describe the variants.

**RESULTS**

A solid, grayish-yellow tumor of upper limb of a 64-year-old male patient measured 1.5 cm. The neoplasm was located in dermis and invaded subcutaneous adipose tissue. Anatomical location of the tumor was superficial partially intradermal and partially subcutaneous (pT1a edition 8th pTNM). The tumor spread in the typical manner for DFSP with subcutaneous infiltration of adipose tissue and without tumor infiltration of the narrow dermal band adjacent under the epidermis. The low grade tumor adhered to areas of high grade sarcoma occupying up to 45% total tumor mass. The tumor was composed of spindle cells which in high grade areas showed enlarged, oval nuclei with marked increase in abnormal mitotic rate (Fig. 1). Median mitotic score was 9 mitoses per 10 HPF, but it ranged from almost none, sparse mitoses in low grade fields of DFSP to 26 mitoses /10HPF of high grade sarcoma. Several immunohistochemical stains were applied including SMA, desmin (-negative), Ki-67 (highly positive reaction (+++) in up to 50% of neoplastic cells in so-called “hot spots” to only 10% in the remaining low grade texture), CD34: reaction (+++) positive exclusively in low grade areas of DFSP and negative in high grade areas of sarcoma (Fig. 2). Masson trichrome histochemical stain produced bluish appearance in the tumor. It was consistent with dermatofibrosarcoma protuberans (ICD-O3: 8832/3) in areas of low mitotic activity, low atypia and sustained CD34 positivity. CD34-negative texture with high mitotic index and atypia was consistent with to high grade sarcoma apparently of fibrous origin, given category of poorly differentiated fibrosarcoma (ICD-O3: 8810/3). The high grade component was graded (G3) and scored according to French Federation of Cancer Centers Sarcoma Group (FNCLCC): total score of 6 points, tumor differentiation: 3 points +, mitotic count: 3 points (up to 26 mitoses/10HPF in high-grade fields), + no necrosis: 0 points. In low grade sarcomatous component ADAMTS20 (NM_025003: c.1661C>T, p.P554L) NF1 (NM_001042492: c.2173G>T, p.E725X) and PKHD1 (NM_138694: c.11074C>T, p.R3692X) were revealed with following allelic frequencies: 25%, 27% and 17%. In high grade sarcomatous component allelic frequencies of the same mentioned mutations were 30%, 30% and 14%, respectively.

**DISCUSSION**

A product of mutated in described case gene ADAMTS-20 belongs to protein of the ADAMTS family which contain disintegrin and metalloproteinase domains, with thrombospondin type-1 repeats. These complex cellular proteases have protumor and antitumor activity. They can be secreted by tumor cells and stromal cells and actively modify local microenviroment. ADAMTS proteases have been found overexpressed, silenced or mutated in different cancers of different origin. This suggest many contexts dependent function in tumor inhibition and promotion as well. According to bioinformatic analysis (Varsome) mutation detected in the presented case was classified as variant of unknown significance (VUS). This mutation to our knowledge was described for the first time and the impact on the ADAMTS activity and expression is not known. In immunohistochemical analysis ADAMTS-20 was found to be down-regulated in regard to cancer progression in colorectal adenocarcinoma. In addition, ADAMTS-9 which share
Figure 1. Morphology of dermal high grade sarcoma arising in dermatofibrosarcoma protuberans. 1a. Interface between high grade and low grade areas of sarcoma marked by high and lower cellularity (H&E ×100). 1b. Storiform pattern of hypercellular areas of the tumor (H&E ×100). 1c. High grade sarcoma texture (H&E ×200). 1d. High grade sarcoma texture with numerous mitoses (H&E ×400).

Figure 2. Immunohistochemistry of dermal high grade sarcoma arising in dermatofibrosarcoma protuberans. 2a. Strong CD34 positivity in areas of DFSP (CD34 staining ×200). 2b. Negativity to CD34 in areas of high grade sarcoma with a few remaining positive interspersed cells (CD34 staining ×200). 2c. Low Ki-67 labeling (MIB index) in areas of DFSP (Ki-67 staining, ×200). 2d. High Ki-67 labeling (MIB index) in high grade sarcoma texture (Ki-67 staining ×400).
high degree similarity with ADAMTS-20 function as a tumor suppressor gene in breast cancer tumor cell lines and is downregulated by promotor methylation. ADAMTS-20, as one of AdamT5-A metalloproteases, can contribute to invasive potential of the tumor, because in the light of findings by Ismat et al. ADAMTS-20 is responsible for cell migration by means of detachment of cells from the extracellular matrix. It cannot be excluded that some mutations of PKHD1 (polycystic kidney and hepatic disease gene 1) gene seem to aggravate dissemination of neoplastic cells, as miR-365-1 modulated PKHD1 counteracted cell-cell adhesion. Mutation detected in our case has been already described in colorectal carcinoma sample. Preclinical studies revealed that Pkhd1 loss in mice promoted intestinal tumorigenesis in APC-defective rodents. In addition, PKHD1 gene silencing induced proliferation, migration and invasion of human intrahepatic cholangiocarcinoma HuCCT-1 cells. On basis of whole-exome sequencing, RNA sequencing and methylation analysis of myxofibrosarcoma, Ogura et al. recognized NF1 as one among potential driver genes like ATRX, Jak1 and NTRK1 as well as novel oncogenic BRAF fusion in this type of sarcoma, which belongs to category fibrous malignancies as DSFP and fibrosarcoma. In addition, a frame-shift alteration in NF1 gene was detected in a case of a pediatric ovarian fibrosarcoma. Generally, NF1 mutations are involved in several tumor types including: Malignant Peripheral Nerve Sheath Tumors, Gastrointestinal Stromal Tumor, Rhabdomyosarcoma.

As our paper has its limitations that it is only one case report, the great care was taken to eliminate categorization of these mutations as stochastic passenger mutations. Presented morphology and immunoprofile diagnosis of DFSP that is why we did not perform FISH analysis to reveal COL1A1-PDGFB fusion. However, lately it has been shown a group of aggressive spindle cell sarcomas of morphology reminiscent of DFSP does not harbour COL1A1-PDGFB fusion but EML4-NTRK3 fusion. Thus, this approach to molecular analysis of dermal fibrosarcoma arising from dermatofibroma is well-founded in the light of mentioned above investigations, but in the first place it is reasoned by the fact that fibrosarcoma is a life threatening malignancy whenever this neoplasm undergoes dissemination via vasculature.

CONCLUSION

None of detected mutations can be regarded as a mutation that would definitely induce phenotype of high malignancy, because ADAMTS20, NF1 and PKHD1 mutations were detected both in high grade fields of fibrosarcoma and in low grade areas of dermatofibrosarcoma protubersans. However, the mutations of these peculiar genes could contribute to migration of neoplastic cells and therefore they could favour invasive potential of the tumor. Our results also show that these mutations could appear at the early stages of tumor development.

REFERENCES


Обзор потенциальной значимости мутаций ADAMTS20, NF1 и PKHD1, обнаруженных при секвенировании следующего поколения (NGS) при дермальной фибросаркоме, возникшей при выбухающей дерматофибросаркоме

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Абстракт

Мы исследовали состояние фибросаркомы, возникшей при выбухающей дерматофибросаркоме у 64-летнего мужчины. Кожная, твёрдая, серо-белая, негативная для десмина окраска с синим трихромом имела диаметр 1,5 см, классифицированная как рT1a (edition 8 pTNM). Она была образована из веретенообразных клеток. Соответствовала выбухающей дерматофибросаркоме (ICD-O3: 8832/3) в областях с низкой митотической активностью, низкой атипичностью и сохранённой позитивностью CD34. CD34-негативная ткань с высоким митотическим индексом и атипичностью соответствовала высокосортной саркоме, по-видимому, фиброзного происхождения, которая была отнесена к категории фибросаркомы. Компонент высокого класса был классифицирован как G3 в соответствии с группами саркомы Французской федерации раковых центров (FNCLCC): общий результат в 6 баллов: дифференцировка опухоли в 3 балла + митотическое количество в 3 балла (до 26 митозов / 10 HPF в высококлассных областях ), + отсутствие некроза: 0 баллов. В низкосортном саркоматозном компоненте ADAMTS20 (NM_025003: c.1661C> T, p.P554L) NF1 (NM_001042492: c. 2173G> T, p.E725X) и PKHD1 (NM_138694: c. 11074C> T, p.R3692X) были установлены следующие частоты аллелей 25%, 27%, 17% и 44%. В компоненте высокого класса аллельные частоты тех же самых упомянутых мутаций были 30%, 30%, 14% и 51% соответственно. Учитывая наши результаты, мы можем утверждать, что ни одна из идентифицированных мутаций не может считаться мутацией, которая, несомненно, индуцировала бы фенотип с высокой злокачественностью, поскольку мутации ADAMTS20, NF1 и PKHD1 также установлены при саркоме высокого класса, и в областях выбухающей дерматофибросаркомы низкого класса. Следует также отметить, что эти мутации возникают на ранних стадиях развития опухоли.

Ключевые слова
ADAMTS20, NF1 и PKHD1, выбухающая дерматофибросаркома, фибросаркома