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Original Article

Identification of a FXIIIA variant in human neuroblastoma cell lines

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Abstract: FXIII is a transglutaminase consisting of two catalytic (FXIIIA) and two non-catalytic subunits (FXIIIB) in plasma, where this enzyme is responsible for stabilizing fibrin clots. Although possible functions of intracellular FXIIIA have been proposed, these remain to be established. We show that a 40 kDa protein species of FXIIIA is present in the human neuroblastoma cell lines SH-SY5Y and LAN5. These data reveal the presence of a new uncharacterised variant of FXIIIA, possibly due to an alternative splicing, in nervous cells.

Keywords: Transglutaminases, cellular factor XIII, post-translational modifications of proteins, CNS

Introduction

Coagulation factor XIIIA (FXIIIA) is a member of the transglutaminase (TGs; EC 2.3.2.13) gene family [1]. Factor XIIIA functions during the blood coagulation process as a proenzyme which catalyses the acyl transfer reaction in the presence of Ca^{++} and thrombin, forming γ-glutamyl-ε-lysyl covalent bonds between polymerised fibrin molecules [2, 3]. The covalently modified fibrin is mechanically stronger, less deformable, and more resistant to lysis by plasmin when compared with the noncross-linked fibrin fiber network.

To date, at least eight different TGs, distributed in the human body, have been identified. In the Nervous System several forms of TGs are simultaneously present [4]. Moreover, several alternative splice variants of TGs, mostly in the 3'-end region, have been identified. In particular TG2, which is the best-studied enzyme of the TG family, shows at least five splice variants [5-8]. Some of these splice variants, interestingly, are differently expressed in neurological pathologies, such as Alzheimer's disease (AD) [5]. A number of previous reports demonstrated that FXIII exists also as an intracellular type, known as "cellular factor XIII (cFXIII)" in various types of cells, such as hepatocytes, monocytes/macrophages, platelets [9-12] and tissues, including lung, kidney, stomach, skin, esophagus, liver, testis, bone and spleen [13, 14]. The variety of tissue distribution of cFXIII, therefore, suggests that catalytically active FXIII subunit (FXIIIA) has another important function in addition to its role in clot formation and wound healing [1].

Several reports have described the presence of Factor XIIIA also in the Nervous System of several species [15, 16], suggesting that the intracellular form of FXIII could be involved in the regenerating processes of this tissue. One of these studies, interestingly, showed that in the rat Nervous System a smaller FXIIIA protein is often present, probably due to the cleavage of a larger protein and its subsequent activation [15]. Curiously, some studies carried out to analyse the presence of the FXIIIA in the human and mouse Nervous System by RT-PCR technique, were not able to identify the FXIIIA mRNA [4, 17].

In the present study the structure of the FXIIIA mRNA expressed in the rat brain by 3'RACE technique was analysed. Subsequently, the presence and the activity of the FXIIIA in human neuroblastoma cell lines were analysed. Interestingly, a 40 kDa protein species of FXIIIA, which can be activated by thrombin, is present in human neuroblastoma cell lines. These data

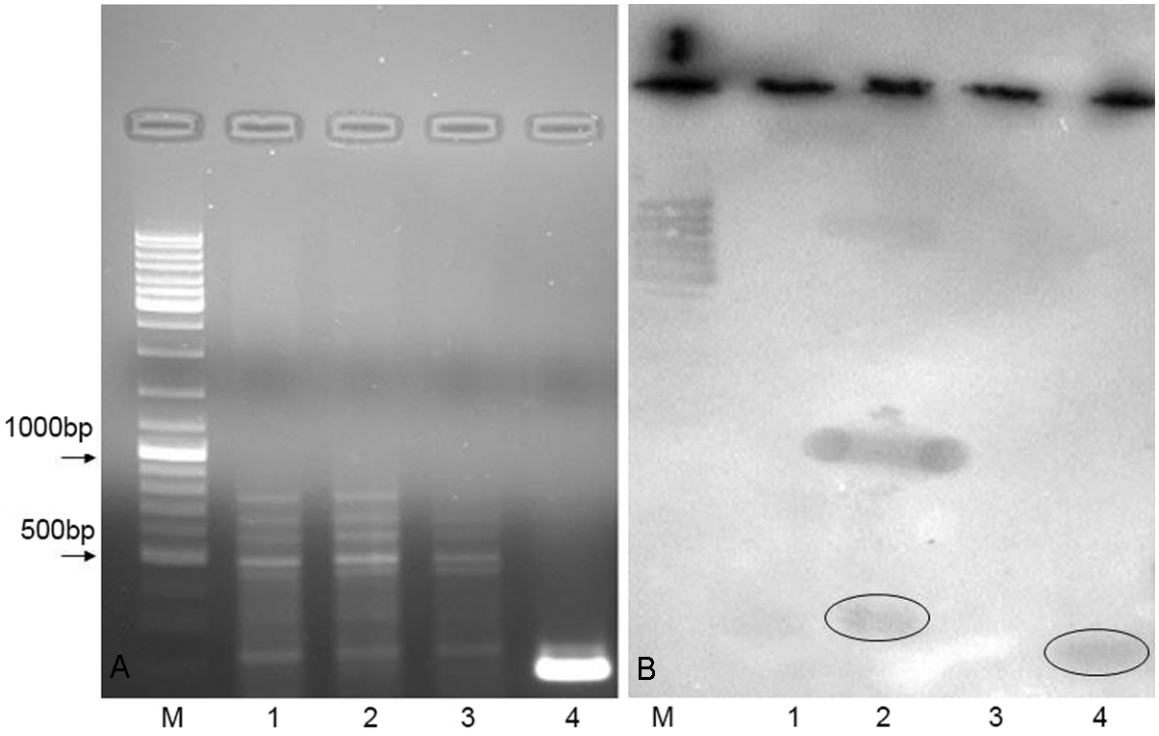

Figure 1. A: 3'-RACE on rat brain mRNA. Lane 1: PCR at 57°C; lane 2: PCR at 60°C; lane 3: PCR at 63°C; lane 4: FXIIIA cDNA (positive control); M: DNA markers. B: Southern blot on 3'-RACE products from rat brain mRNA. Lane 1: PCR at 57°C; lane 2: PCR at 60°C; lane 3: PCR at 63°C; lane 4: FXIIIA cDNA (positive control); M: DNA markers.

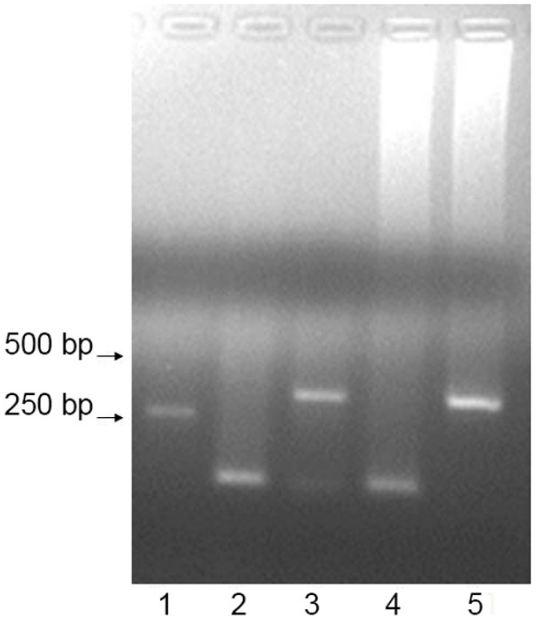

Figure 2. RT-PCR analysis on human neuroblastoma cells SH-SY5Y. Lane 1: GAPDH; lane 2: amplicon of 178 bp on FXIIIA mRNA; lane 3: amplicon of 329 bp on FXIIIA mRNA; lane 4: amplicon of 178 bp on plasmid containing human FXIIIA; lane 5: amplicon of 329 bp on plasmid containing human FXIIIA.

suggest that a new variant of intracellular transglutaminase FXIIIA is present in nervous cells.

Materials and methods

Materials

N,N-dimethylated bovine milk casein (*Mr*; 35,000) was purchased from Sigma (St. Louis, MO). [^{14}C]spermine tetrahydrochloride ($(H_2NCH_2$-$CH_2CH_2NH^{14}CH_2CH_2)_2$ + 4HCl) (SPM; sp act 114 mCi/mmol) was purchased from Amersham (Little Chalfont, Buckinghamshire, UK). Rabbit anti-human FXIIIA polyclonal IgG was purchased from Sigma (St. Louis, MO). Thrombin was purchased from Instrumentation Laboratories (Bedford, MA, U.S.A.).

Cell culture

Human neuroblastoma cells SH-SY5Y were grown in DMEM: Ham's F12 supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin. Human neuroblastoma cells LAN5 were grown in RPMI 1640 supplemented with 10% fetal

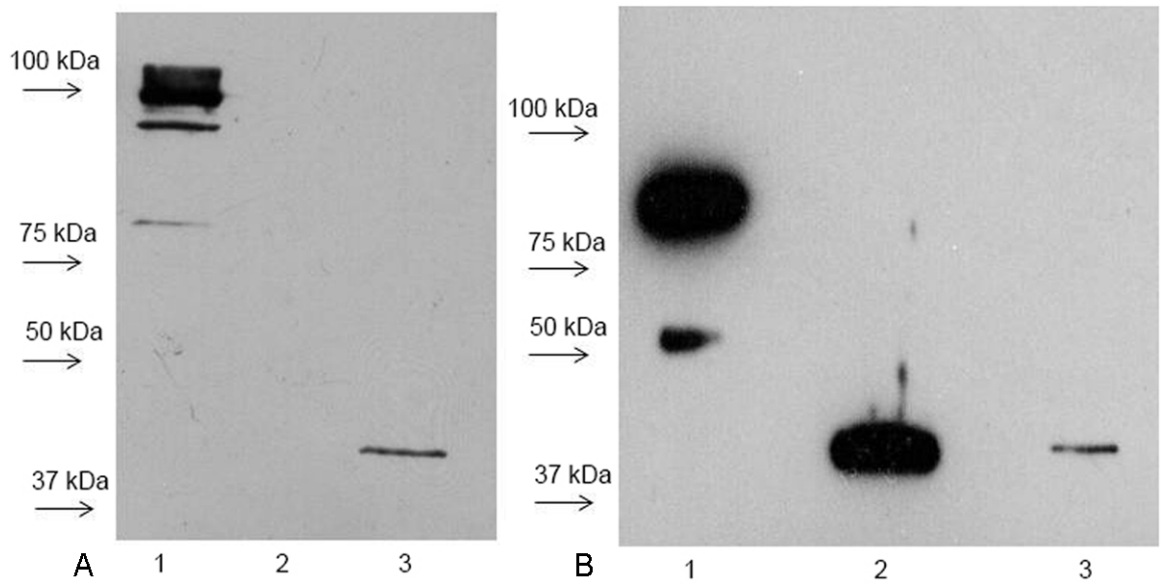

Figure 3. Western blot analysis on cytosolic proteins from human neuroblastoma cell lines SH-SY5Y (A) and LAN5 (B). A: Lane 1: 20 mg of human plasma FXIIIA; lane 2: 1 mg of human plasma FXIIIA; lane 3: 80 mg of cytosolic proteins from human neuroblastoma cell line SH-SY5Y. B: Lane 1: 20 µg of human plasma FXIIIA; lane 2: 48 µg of total homogenate proteins from human neuroblastoma cell line LAN5; lane 3: 8 µg of cytosolic proteins from human neuroblastoma cell line LAN5.

bovine serum, 50 U/ml penicillin and 50 µg/ml streptomycin. Cells were grown at 37°C in a humidified atmosphere containing 5% CO_2.

mRNA extraction and RT-PCR analyses

Adult Sprague Dawley rats of both sex were killed in accordance with the regulations of the Italian Home Office (animals bread in house). RNA extraction from rat brains and from cell cultures, mRNA purification and reverse transcription were performed by using respectively the Chomczynski and Sacchi protocol [18], the mRNA miniprep kit Genelute (Sigma) and Reverse Transcriptase kit (Fermentas). 3-RACE was performed on rat brain mRNA using the kit FirstChioce RLM (Ambion) under the conditions recommended by the manufacturer and by using, as rat FXIIIA gene specific primers, the following sequences: 5'-GCCCCATTTGTTTTTGC AGAGGTCAAC-3' (sense); 5'-GCCCTCCTGAAATTT GTAAGTAT-3' (antisense). PCR products were confirmed by using a biotinylated probe made by the same primers on the rat FXIIIA mRNA. Primers used to amplify cDNA made from human neuroblastoma cell line SH-SY5Y were the followings: 5'-CCTTGTAAAGTCAAAAATGTCA GAAACTTCC-3' (sense) and 5'-CGTATTCCACCC TGAAGAGATCCC-3' (antisense), localized on exons 1 and 4 of human placental FXIIIA mRNA (amplicon size 329 bp); 5'-GCACCTTTTGTTT TTGCAGAGGTCAACAGCGAC-3' (sense) and 5'-G-ACCTTCTTGGAATTTGTAAGTATCAG-3' (antisense), localized on exons 10 and 11 of the human FXIIIA mRNA (amplicon size: 178 bp), corresponding to the putative calcium binding domain. As positive control, a plasmid containing the FXIIIA full length cDNA from human placenta (generous gift of dr. A. Belkin, U.S.A.) was used. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA was used as internal control.

Western blot analysis

The cells were washed twice with PBS and homogenized in a buffer containing 100 mM Tris-HCl (pH 7.4), 5 mM EDTA, 5 mg/ml leupeptin and 2 mM $PhMeso_2F$. Total cell homogenate and cytosolic fraction obtained after centrifugation at 18,000 *g*, were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10-15% acrylamide slab gels. After electrophoresis, proteins were transferred to a nitrocellulose membrane for 2.5 h at 200 mA (in Tris-glycine). The immune reaction was carried out as follows: the blot was incubated overnight in PBS containing 5% skim milk at 4°C and then with the rabbit polyclonal antibody anti-human Factor

Table 1. Transglutaminase activity analysis in immunoprecipitate obtained from human neuroblastom a cell line SH-SY5Y

	+EDTA	+Calcium	+Calcium +Thrombin
Specific activity (pmol Spm/2 h/μg of proteins)	N.D.	N.D.	7.5

XIIIA (Sigma-Aldrich) (diluted 1:1,000) in PBS containing 5% skim milk for 2 h at 37°C. This was followed by several washings with PBS containing 0.05% Tween-20, incubation with 1:10,000 peroxidase-conjugated goat anti-rabbit IgG (Sigma-Aldrich) for 1 h at room temperature, several washings with PBS containing 0.05% Tween-20, and development with the chemiluminescence technique ECL detection system (Amersham, Arlington Heights, Ill.) for 1 min. Each Western blot analysis was repeated at least three times. Purified human plasma FXIIIA (Zedira, Germany) was used as positive control.

Immunoprecipitation protocol

Cytosolic protein extracts (200 μg) were subjected to immunoprecipitation with 0.5 μg of the rabbit polyclonal antibody anti-human Factor XIIIA (Sigma-Aldrich) for 1 h at 4°C. Immune complexes were collected with 50 μl of protein A-sepharose overnight at 4°C under agitation. The protein A-sepharose/immune complex was washed four times with cold PBS, resuspended in 20 ml of SDS-loading buffer, heated to 95°C for 5 min, centrifuged and the supernatants electrophoresed by 10% SDS-PAGE and the protein was electro-transferred on a nitrocellulose film.

The protein was detected using the rabbit polyclonal antibody anti-human Factor XIIIA (Sigma-Aldrich) (diluted 1:1,000) and then with a secondary anti-rabbit HRP-conjugated monoclonal antibody (diluted 1:2,000). The film was washed with TBS containing 0.05% Tween 20 and the specific reactivity was detected by the chemiluminescence technique ECL detection system (Amersham, Arlington Heights, Ill).

Thrombin-dependent transglutaminase activity assay

Transglutaminase activity was assayed by measuring the incorporation of spermine (SPM) into N,N-dimethylated casein on preparations of subcellular fractions of cell homogenates, obtained after centrifugation at 18,000 *g* and immunoprecipitation . The reaction mixture contained 100 mM Tris-HCl (pH 7.4), 10 mM DTT, 2.5 mM $CaCl_2$, 0.075 mM [^{14}C]-labeled SPM (114 Ci/mmol) (Amersham, Life Sciences), 4 mg/ml N,N-dimethylated casein (Sigma) and thrombin (20 U) in a final volume of 100 μl. The reaction mixture was incubated for 2 hrs at 37°C and placed on ice. Cold trichloroacetic acid (TCA) containing 1 mM SPM was added to a final concentration of 10%. Samples were centrifuged (14,000 *g* for 10 min at room temperature) and the pellet was washed twice with 1 ml of 10% TCA containing 1 mM SPM. Samples were dried and resuspended in 200 μl of 0.1 N NaOH. Radioactivity was measured in 5 ml of scintillation cocktail PicoFlour 30 (United Technologies/Packard). All activity assays were performed at least two times, each experiment in duplicate.

Results and discussion

FXIIIA mRNA analysis in rat brain by 3'-RACE technique

To analyse the 3'-end region of the FXIIIA mRNA in rat brain, a 3'-RACE protocol was performed on mRNA isolated from rat brain. As positive internal control, an amplicon of 178 bp, localized on exons 10 and 11 of the rat FXIIIA mRNA, was amplified (**Figure 1A**, lane 4). To identify the FXIIIA amplicon among the several products obtained in the 3'-RACE protocol performed at different temperatures of annealing, a biotinylated probe, made by the same primers used to amplify the FXIIIA amplicon as positive internal control, was used. As shown in **Figure 1B**, a positive signal of approx. 300 bp was identified in lane 2, instead of a product of 2200 bp, as it was expected. These data suggest that the FXIIIA mRNA in the rat brain has a different structure in the 3'-end region, probably due to an alternative splicing process.

RT-PCR analysis for FXIIIA in human neuroblastoma cells SH-SY5Y

To analyse the expression of FXIIIA mRNA in human neuroblastoma cells SH-SY5Y, RT-PCR protocol was performed. As shown in **Figure 2**, the sizes of the amplicons of FXIIIA obtained from human neuroblastoma cells SH-SY5Y

(**Figure 2**, lanes 2 and 3) were the same as those obtained from the positive controls carried out with a plasmid containing the human placenta FXIIIA cDNA (**Figure 2**, lanes 4 and 5). These data suggest that human neuroblastoma cells SH-SY5Y express FXIIIA mRNA, whose structure in the 5'-end region and in the putative calcium binding region corresponds to that of FXIIIA mRNA from human placenta.

Western blot analysis for FXIIIA in human neuroblastoma cells SH-SY5Y and LAN5

The presence of FXIIIA in human neuroblastoma cell lines SH-SY5Y and LAN5 was examined also by Western blot analysis. To confirm the specificity of our rabbit polyclonal antibody anti-human Factor XIIIA used in this analysis, the antibody was tested against purified human plasma FXIIIA as positive control. As shown in **Figure 3**, the antibody detected only a 40 kDa antigen in the cytosolic fractions of human neuroblastoma cells SH-SY5Y and LAN5 (**Figure 3A** and **3B**, lane 3), while the positive control of 80 KDa was detected in the lane containing purified human plasma FXIIIA (**Figure 3A** and **3B**, lane 1).

TG activity in immunoprecipitate from human neuroblastoma cells SH-SY5Y

To investigate whether the 40 kDa protein species of FXIIIA in human neuroblastoma cells SH-SY5Y possesses catalytic activity, an immunoprecipitation protocol was carried out to isolate the FXIIIA from other cytosolic proteins of human neuroblastoma cells SH-SY5Y. The immunoprecipitate was confirmed by Western blot analysis by using the rabbit polyclonal antibody anti-human Factor XIIIA (data not shown), and then used for TG activity assay. As shown in **Table 1**, immunoprecipitate of FXIIIA from human neuroblastoma cell line SH-SY5Y didn't show any TG activity in presence of 2.5 mM Ca^{++}, while a significant TG activity was shown in presence of Ca^{++} and thrombin (20 U). These data show that the 40 kDa protein species of cFXIIIA, present in human neuroblastoma cell line SH-SY5Y, has not been already proteotically activated by thrombin, but its TG catalytic activity appears only in presence of thrombin. Furthermore, its reduced size (40 kDa) seems not to be due to a proteolytic process, since its enzymatic activity is absent without thrombin, but, more likely to a splicing process, involving the 3'-end region of the cFXIIIA mRNA.

In conclusion, taken together, these data suggest that a new variant of cFXIIIA is present in rat brain and human neuroblastoma cell lines SH-SY5Y and LAN5, probably due to a splicing process involving the 3'-end region of the cFXIIIA mRNA. Further studies should be carried out to clarify the physiological role of this intracellular FXIIIa variant, since previous works have suggested that the presence of Factor XIIIA in the Nervous System of several species could be involved in the regenerating processes of this tissue [16, 17].

Abbreviations

TGs, transglutaminases; FXIII, Factor XIII; AD, Alzheimer's disease; PBS, Phosphate Buffered Saline; TBS, Tris Buffered Saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SPM, spermine; DTT, ditiothreitol; TCA, Trichloroacetic acid; RACE, rapid amplification of cDNA ends.

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Disclosure of conflict of interest

There is no conflict of interest.

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