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Elevated levels of IL-1 β in Fanconi Anemia group A patients due to a constitutively active PI3K-AKT pathway are capable of promoting tumor cell proliferation.

Short title: IL-1 β is overproduced in FA-A patients

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ABSTRACT

Fanconi Anemia is a hereditary disease characterized by congenital malformations, progressive bone marrow failure and an extraordinary elevated predisposition to develop cancer. In the present article we describe an anomalous high level of the proinflammatory cytokine IL-1 β present in the serum of Fanconi Anemia patients. The elevated levels of IL-1 β were completely reverted by transduction of a wild type copy of the FancA cDNA into FA-A lymphocytes. Although the transcription factor NF- κ B is a well established regulator of IL-1 β expression, our experiments did not show any proof of elevated NF- κ B activity in FA-A cells. However, we found that the overexpression of IL-1 β in FA-A cells is related to a constitutively activated PI3K-AKT pathway in these cells. We provide evidence that the effect of AKT on IL-1 β activation is mediated by the inhibition of GSK3 β . Finally, our data indicate that the levels of IL-1 β produced by FA-A lymphoblasts are enough to promote an activation of the cell cycle in primary glioblastoma progenitor cells. Together, these data demonstrate that the constitutive activation of the PI3K-AKT pathway in FA cells upregulates the expression of IL-1 β through a NF- κ B-independent mechanism and that this overproduction activates the proliferation of tumor cells.

KEY WORDS

Fanconi Anemia (FA), Interleukin-1 beta (IL-1 β), AKT, Glycogen Synthase Kinase 3 beta (GSK3 β), NF- κ B, β -catenin.

ABBREVIATIONS

C/EBP, CCAAT/enhancer-binding protein, ERK, extracellular-signal-regulated kinase, FA, Fanconi Anemia, GSK3, glycogen synthase kinase 3, HSF-1, Heat Shock Factor 1, IL-1 β , interleukin-1 beta, JNK, c-Jun N-terminal kinase, NF- κ B, nuclear factor- κ B, PI3K, phosphatidylinositol (phosphoinositide) 3-kinase, RT, reverse transcription, TNF α , tumor necrosis factor alpha.

INTRODUCTION

Fanconi Anemia is a rare recessive genetic disease characterized by congenital malformations, high chromosomal fragility, progressive bone marrow depletion and a striking susceptibility to develop cancer [1]. The disease is genetically heterogeneous and is caused by mutations in at least 13 different genes that correspond to 13 different complementation groups. Nine of these proteins aggregate in response to damage in the DNA and monoubiquitinate the protein FANCD2, which then relocalizes into the points of damage together with other DNA repair proteins. However, not every aspect of the cellular FA phenotype could be explained through the formation of this nuclear complex. A growing amount of evidence points to a role of FANCC and FANCA in the signal transduction pathways leading to the inhibition of apoptosis and the promotion of cell survival [2-4]. Mutation analysis of FANCC identified different domains responsible for its separated nuclear and cytoplasmic functions [5]. The deficiency in different FANCC proteins has been associated with alterations in the activation of several transduction pathways [2, 6, 7]. A large effort has attempted to discover alterations leading to the hematological problems of these patients. However, further investigation is needed to fully understand the molecular basis for the cancer propensity of FA. Several groups, including ours, are trying to approach this problem by defining a molecular profile of the genes transcriptionally altered in FA. Interestingly, the expression profile of FANCC-deficient cells unveiled an unsuspected anti-inflammatory role for the FA proteins [8]. In line with this, other authors have reported an exacerbated inflammatory response in FANCC-deficient mice [9]. In addition, TNF α , a well known pro-inflammatory cytokine, has been shown to be oversecreted in FA patients [10]. All these observations suggest that some cytokines and immune modulators may help explain the clinical features of FA patients, including bone marrow failure and increased tumor susceptibility.

The association between chronic inflammation and cancer has been known for some time and is supported by animal models [11-13]. IL-1 β and TNF α are two of the best known pro-inflammatory cytokines. The secretion of IL-1 β by tumors has been associated to a more aggressive phenotype and poor outcome [14]. Consistently, tumors actively producing IL-1 β grow faster, are more metastatic, promote tumor angiogenesis and more easily elude the immune response [15]. Furthermore, IL-1 β transgenic mice, or mice bearing a targeted deletion of the IL-1Ra gene that codes for the naturally occurring antagonist of the IL-1 β receptor, show an increased incidence of tumors [16, 17].

Patients of the group A (FA-A) are the most abundant among the Fanconi Anemia patients. However, there is very little literature regarding the role of cytokines or other modulators of the immune and inflammatory response in this group of patients. Our massive gene expression studies showed that IL-1 β is the most prevalent overexpressed gene in FA-A cells. In addition, we studied the molecular mechanisms leading to this overexpression and present *in vitro* evidence for the activity of the IL-1 β secreted by FA-A cells in tumor cell proliferation.

EXPERIMENTAL

Cell culture and ELISA analysis.

Peripheral blood mononuclear cells were obtained from healthy donors and FA patients after informed consent. To determine the complementation group of these patients, cells were transduced with retroviral vectors expressing FANCA, FANCC, FANCF, or FANCG proteins (kindly provided by H. Hanenberg, Heinrich-Heine University, Dusseldorf, Germany) and then exposed to increasing concentrations of MMC. Epstein-Barr virus (EBV)-transformed lymphoblasts from FA-A patients and healthy donors were maintained in RPMI media (Biochrom, Berlin, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS) and antibiotics. Cells were grown in the presence of LY294002 (Alexis, San Diego, USA) or BAY 11-7082 (Sigma, Saint Louis, USA) where indicated and the amount of IL-1 β and TNF α in the culture medium was determined by ELISA (BD, San Diego, USA) following the manufacturer's instructions. The same ELISA assay was used to determine the amount of IL-1 β in the serum of the blood samples obtained from FA patients and healthy donors after informed consent.

RT-PCR analysis

Total RNA was prepared using TRI reagent (MRC, Cincinnati, USA). To assess mRNA expression, a semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) method was used. For the RT reaction, RNA (10 μ g) was primed with random hexamers and reverse transcribed with Superscript MMLV reverse transcriptase (Invitrogen, Carlsbad, USA) in a 20 μ l volume following the manufacturer's instructions. The generated cDNA was amplified using primers for human IL-1 β (5'-TCATTGCTCAAGTGTCTGAAGC and 5'-TGGTCGGAGATTCGTAGC), FancA (5'-GCTCTTCAGGAATCTGTGCTGC and 5'-TACAGTGAGGTGAGCAGAGG), A1 (5'-CGGATGTGGATACCTATAAGG and 5'-GTCATCCAGCCAGATTTAGG), IAP1 (5'-TGAGCATGCAGACACATGC and 5'-TGACGGATGAACTCCTGTCC), XIAP (5'-TGGCAATATGGAGACTCAGC and 5'-TGCACTTGGTCACCAATACC), Bcl-2 (5'-AGATGTCCAGCCAGCTGCACCTGAC and 5'-AGATAGGCACCCAGGGTGATGCAAGCT) and β 2 microglobulin (β 2 μ) (5'-GAGACATGTAAGCAGCATCA and 5'-AGCAACCTGCTCAGATACAT). After 30 amplification cycles, except for β 2 μ (25 cycles), the expected PCR products were size-fractionated onto a 2% agarose gel and stained with ethidium bromide.

Western blot analysis

To prepare whole cell lysates, cells were collected by centrifugation and washed once with PBS and then lysed in EBC buffer (50mM Tris [pH 8.0], 120 mM NaCl, 0.5% Nonidet P-40) supplemented with aprotinin (11.5 μ g/ml), leupeptin (11.5 μ g/ml) phenylmethylsulfonyl fluoride (50 μ g/ml), NaF (100mM) and Na Orthovanadate (0.2 mM). Protein concentration was determined by BCA following the manufacturer's instructions (Pierce, Rockford, USA). Proteins (100 μ g) were resolved in SDS-PAGE and transferred to PVDF filters. Blots were incubated with rabbit antibodies against proIL-1 β , (Santa Cruz, Santa Cruz, USA), phospho-AKT (Ser 473), AKT (Cell Signaling, Danvers, USA) or mouse anti- α -tubulin antibody (Sigma, Saint Louis, USA) and then incubated with goat antirabbit or antimouse antibodies conjugated with horseradish peroxidase (Pierce,

Rockford, USA). Bound antibody was detected by a chemiluminescence assay (Pierce, Rockford, USA).

Electrophoretic mobility shift assay (EMSA)

Nuclear protein extracts were obtained from different cell lines as previously described [18]. The nuclear extracts (5–10 μ g of total protein) were incubated on ice for 15 min with 1 μ g of poly(dI-dC) (Pharmacia) in 2.5% glycerol–10 mM Tris (pH 7.5)–50 mM NaCl–1 mM EDTA–1 mM dithiothreitol–1 mM phenylmethylsulfonyl fluoride–0.5 μ g of bovine serum albumin per ml. Antibodies or competitor oligonucleotides were added during this incubation. Upon addition of 100,000 cpm of the 32 P-labeled double stranded DNA probe from the promoter region of the IL-1 β gene containing a NF-kappaB (NF-kB) responsible element (5' -GTAACGTGGGAAAATCCAGTATT), the reaction mixture was incubated for 15 min on ice and then resolved on a 5% polyacrylamide gel run at 25 mA for 2.5 h. The gel was dried and exposed to X-ray film.

Cocultures and cell cycle analysis

125,000 glioblastoma progenitor cells were plated in six well plates and cultured in DMEM-HAM's medium supplemented with 2% B27, 20 ng/ml EGF, 20 ng/ml bFGF and 180 units/ml heparin, overnight. Coculture canisters were then set on the corresponding wells containing 10⁶ FA-A lymphoblasts in the same medium, in the presence or absence of 75 μ g/ml of IL-1Ra. One of the wells was cultured in the presence of 20 ng/ml of recombinant IL-1 β as a control. After 36 hours, the glioblastoma stem cells were harvested and their cell cycle analyzed by propidium iodide staining on a flow cytometer.

Gene reporter assay

A genomic PCR fragment of 990 base pairs (bp) (-950 to +40, referred to the initiation of transcription) from the 5' regulatory region of IL-1 β (IL-1 β -950kb) was amplified with specific primers and cloned into the pGL2basic luciferase reporter vector (Promega, Madison, USA). Deletions of this original construct were obtained by amplification using specific primers and cloning into pGL2basic. HCT116 cells were cotransfected with 2 μ g pGL2–IL-1 β constructs and 0.1 μ g pRSV– β -gal in triplicate using lipofectamin (Invitrogen, Carlsbad, USA) according to the manufacturer's directions. 36 hours after transfection, cell extracts were prepared and analyzed for their luciferase activity by using a reporter gene assay system (Promega, Madison, USA). Results were normalized for transfection efficiency with the β -galactosidase activity values obtained in the same extracts.

RESULTS

IL-1 β is overexpressed in FA patients

Our lab has focused on the study of the gene expression profile of FA cells responsible for their characteristic phenotype. In order to have a global picture of this expression profile we carried out a high throughput analysis of RNA obtained from primary blood samples of FA-A patients (RNA from three different FA patients and three healthy age-matched persons were analyzed independently with Affymetrix U133 Genechip arrays). This analysis revealed several inflammatory cytokines overexpressed in FA-A mononuclear blood cells. IL-1 β was the most overexpressed gene in FA mononuclear cells, 15 fold higher than the healthy counterparts (figure 1A). IL-8 (7.35 fold) and interferon gamma (4.81 fold) were also overexpressed in FA-A cells. Interestingly, the array analysis did not show any increase in TNF alpha mRNA in FA-A cells, although TNF alpha-interacting protein 3 showed a 4.18 fold higher expression compared to healthy donors. Since IL-1 β was the most overexpressed gene in FA-A blood cells, we focused our studies on this cytokine. As shown in figure 1B, the semiquantitative RT-PCR analysis confirmed a significant accumulation of IL-1 β mRNA in FA-A primary samples compared to the control counterparts. We also wanted to check the expression of this cytokine in the Epstein-Barr virus (EBV)-immortalized B lymphoblasts cell lines derived from FA-A patients that we normally use in our studies. Figure 1C confirms the overexpression of IL-1 β in FA-A lymphoblast cell lines.

IL-1 β is synthesized as an immature molecule, pro-IL-1 β , that is digested by caspase-1 to produce its active form that is then secreted to the medium. Interestingly the microarray analysis showed a reduced caspase 1 mRNA accumulation in FA-A cells (2.4 mean fold reduction relative to control cells, see figure 1A). We analyzed whole cell extracts for the pro-IL-1 β level in FA-A lymphoblastoid cell lines. The western blot analysis revealed a 33 kDa band corresponding to pro-IL-1 β of increased intensity in FA-A cell lines (figure 1D). Moreover, the secreted IL-1 β levels in the growing medium of FA-A lymphoblasts were also elevated as analyzed by ELISA (figure 1E). In order to extend our findings to the patients, we analyzed serum samples obtained from patients of different complementation groups. Thus, a total of 17 serums from 12 FA-A patients, 2 FA-D2, 2 FA-G and 1 FA-J patients, were analyzed. Ten out of twelve FA-A patients studied showed an increase in IL-1 β secretion above the mean value obtained from eight controls. Notably, all of the non-A FA patient samples contained an increased level of IL-1 β (figure 1F). Overall these data demonstrate an elevated IL-1 β secretion in FA-A patients most likely due to a transcriptional process since the messenger stability does not seem to be altered (figure 2A).

The deficiency in FANCA is responsible for the overexpression of IL-1 β .

In order to ascertain the involvement of the FancA gene in IL-1 β overexpression, we transduced stimulated primary T lymphocytes with viral expression vectors containing the normal FancA gene. Overexpression of the transduced gene was confirmed by RT-

PCR (figure 2A). As previously seen in B lymphocytes, primary FA-A T lymphocytes derived from two different patients showed increased accumulation of IL-1 β mRNA as compared to control samples or control T cells transduced with a GFP expression viral vector (figure 2A). Transduction of two different viral vectors carrying the FancA ORF into FA-A deficient T cells completely abolished their expression of the IL-1 β mRNA (figure 2A). We obtained similar results when B-lymphoblastoid cells lines derived from FA-A patients were transduced with a viral vector containing the FancA wild type cDNA (figure 2B). The transduction of the FancA cDNA reverted the MMC sensitivity of the FA-A lymphoblasts, confirming the functional expression of the wild type FancA mRNA (data not shown). These data demonstrate that the alteration in the FancA gene is responsible for the overexpression of IL-1 β in FA-A cells.

NF- κ B does not seem to be involved in the overexpression of IL-1 β in FA-A lymphoblasts.

As stated previously, NF- κ B is a main regulator of IL-1 β expression. A constitutive activation of NF- κ B in FA-A lymphoblasts was then conceivable as responsible for the overexpression of IL-1 β . We tested this possibility by analyzing the expression of other NF- κ B target genes in these cells. Figure 3A shows that genes as A1, IAP1, Bcl2 or XIAP genes are expressed at similar levels in control and FA cells. We then tested the binding of NF- κ B to a consensus probe in an EMSA assay, as a measure of its activation in FA cells. NF- κ B bound more weakly to its consensus binding sequence in FA lymphoblasts than in their control counterparts, suggesting a lower level of activation of the NF- κ B pathway (figure 3B). Similar results were found in FA-A fibroblasts (data not shown). In addition to this, we observed that the treatment of FA-A lymphoblasts with BAY 11-7082, a specific inhibitor of the NF- κ B pathway did not inhibit the accumulation of the IL-1 β mRNA in those cells (figure 3C). We also tested a possible alteration in the NF- κ B responsive element of the IL-1 β gene in FA-A cells that may affect its transcription. However, sequencing of a fragment of the 5' regulatory region embracing the NF- κ B element at position -290 [19] cloned from FA-A lymphoblasts or control cells showed no differences (data not shown). All these observations rule out a functional overactivation of the NF- κ B in FA-A lymphoblasts.

The PI3K-AKT pathway is constitutively active in FA-A cells and is related to the overexpression of IL-1 β .

We decided to investigate other possible pathways regulating IL-1 β transcription. MAPK/SAPK (mitogen and stress activated kinases) are kinases with a known influence in the expression and secretion of IL-1 β . However, neither the ERK nor the JNK pathways show an increase in their basal activity in FA-A lymphoblasts, as we have previously reported [2]. An analysis of the phosphorylation status of ERK in these cells with specific anti-phospho antibodies confirmed our previous results (data not shown). All these observations made us discard an involvement of the ERK or JNK pathways in the overexpression of IL-1 β in FA-A cells.

The PI3K-AKT signaling pathway is crucial to many aspects of cell growth and survival, especially in cells under stress conditions. We analyzed the phosphorylation status of AKT in whole cell extracts from FA-A lymphoblasts with an anti-phospho-serine 473-AKT antibody. The western blot analysis depicted in figure 4A shows that AKT phosphorylation was undetectable in control lymphoblasts while it was strongly activated in FA-A lymphocytes. This activation was reverted, at least in part, in FA-A lymphoblasts cultured in the presence of 20 μ M LY294002, an inhibitor of PI3K. More importantly, when FA-A lymphoblasts were phenotypically reverted by transduction of the FancA cDNA, the phosphorylation of AKT was clearly reduced (figure 4B), supporting the idea that the deficiency in the FANCA protein is responsible for the constitutive activation of AKT in FA lymphoblasts. To test if the activated PI3K-AKT pathway is related to the overexpression of IL-1 β in FA-A lymphoblasts, we analyzed the expression of IL-1 β in cells grown in the presence of increasing doses of LY294002. As shown in figure 4C, the treatment with LY294002 induced a dose-dependent reduction of the IL-1 β mRNA expression in FA-A lymphoblasts, almost completely reverting its overexpression. Consistently, the IL-1 β secreted by these cells was also reduced in the presence of the inhibitor (figure 4D). These data demonstrate an involvement of PI3K-AKT pathway in the overexpression of IL-1 β in FA-A lymphoblasts.

The inhibition of GSK3 β by the constitutively activated AKT1 in FA-A lymphoblasts mediates IL-1 β overexpression in these cells.

Among the different proteins regulated by AKT phosphorylation, the inhibitory kinase glycogen synthase kinase 3 (GSK3) caught our eye because of its known role in the regulation of transcription factors involved in IL-1 β gene expression. Whole cell extracts from lymphoblasts were analyzed by western blotting with an anti-phospho-serine 9-GSK3 antibody. As seen in figure 5A, both control and FA-A cell lines showed similar phosphorylation levels of GSK3 α , but GSK3 β was clearly more phosphorylated in FA-A cells at serine 9, suggesting an inhibition of GSK3 β by AKT1 phosphorylation in these cells. In order to confirm a functional inhibition of GSK3 by AKT1 in FA-A lymphoblasts, we analyzed whether the phosphorylation of GSK3 in these cells affects the stability of the β -catenin protein. The western blot depicted in figure 5A shows that inhibition of GSK3 in FA-A lymphoblasts is actually followed by an increase in the levels of β -catenin.

To the best of our knowledge, no reports have described an effect of GSK3 on the modulation of IL-1 β gene expression. To test if IL-1 β is actually regulated by GSK3, we treated control lymphoblasts with increasing doses of Li⁺, a known inhibitor of GSK3 function [20]. Inhibition of GSK3 function by Li⁺ in control lymphoblasts induced a dose response increase in IL-1 β mRNA accumulation reaching the levels observed in FA-A lymphoblasts (figure 5B). In order to provide more evidence supporting this observation, we transfected a dominant negative form of GSK3 β [21] carrying an inactivating mutation in the ATP binding site (GSK3 β -K85A) into control lymphoblasts. The dominant negative transfected cells showed a noticeable increase in the expression of IL-1 β

mRNA compared to the empty vector-transfected cells, further confirming the regulation of the expression of this gene by GSK3 β (figures 5C and D). We also tested the activation of a transfected luciferase construct containing 950bp of the 5' regulatory region of the IL-1 β gene in response to the inhibition of GSK3. We chose HCT116 cells for their high transfection efficiency. Li⁺ induced a 2 fold increase in the IL-1 β promoter activity in these cells (figure 5E). Deletion of the region between positions -350 and -150 decreased the basal activity to half, but the -150 construct was still activated by Li⁺. However, deletion from position -150 to -100 produced a profound decrease in the induction by GSK3 β inhibition of the IL-1 β regulatory region (figure 5E). These are the first observations indicating that GSK3 β is able to modulate the expression of IL-1 β by the inhibition of its transcription, and suggest the loss of this repression as the mechanism mediating the overproduction of IL-1 β in FA-A lymphoblasts.

The IL-1 β produced by FA-A cells is functionally active.

We then asked whether the IL-1 β levels produced by our FancA-deficient cells were enough to promote the growth of cancer cells. To this end, we cultivated primary glioblastoma progenitor cells with FA lymphoblasts separated by a semipermeable membrane, and analyzed the activation of NF- κ B. There have been reported mutations in FANC proteins in a percentage of spontaneous gliomas, so we thought this primary tumor could be suitable for these experiments [22]. EMSA experiments showed an increased binding to the NF- κ B probe in nuclear extracts of glioblastoma cells cocultivated with FA-A lymphoblasts (figure 6A). Thus, the IL-1 β produced by FA lymphoblasts activates a well known pathway involved in survival and proliferation in glioblastoma progenitor cells in vitro. Similar results were obtained with extracts obtained from the mieloblastic leukemia cell line HL60 cocultured with FA-A lymphoblasts (data not shown).

To prove that the IL-1 β levels produced by FA lymphoblasts are enough to induce proliferation of cancer cells, we analyzed the cell cycle of the primary glioblastoma progenitor cells cocultured with FA-A lymphoblast. We detected an increase in the number of cells entering mitosis from 5.3% to 8.3%, similarly to when glioblastoma cells were grown in the presence of 20 ng/ml of IL-1 β , (from 5.3% to 9.8%), (figure 6B, compare panels 1-2 and 3-5). In order to assess that this effect is due to the IL-1 β produced by the FA-A lymphoblasts, we added the naturally occurring IL-1 β competitor, IL-1Ra, to the coculture medium. The addition of IL-1Ra decreased the number of glioblastoma cells entering mitosis from 8.3% to 6.7% (Figure 6B, compare panels 5 and 6), and this matched with a reduction in the activation of NF- κ B in the cells (figure 6A), demonstrating that the increase in proliferating glioblastoma cells was due to the IL-1 β produced by the FA-A cells. A similar activation in cell cycle was observed when HL60 cells were used in the coculture experiments (figure 6C)

To further investigate the functionality of the IL-1 β levels produced by the FA lymphoblasts, we used an oral squamous cell carcinoma cell line derived from FA patients (EUFA1365) [23] in the same kind of coculture experiments. The main solid

tumor affecting FA patients is squamous cell carcinoma, so the use of this cell line that also bears a mutation in the FancA gene, is of special interest to study the effect of IL-1 β in its growth rate. We could quantify a 1.6 fold increase in the percentage of EUFA1365 cells entering mitosis when cocultured with FA-A lymphoblasts compared to the same cells cultured in the presence of control lymphoblasts (Figure 6D). These results support the in vitro functional activity of the IL-1 β levels produced by FA lymphoblasts.

DISCUSSION

Fanconi Anemia is characterized by a progressive bone marrow depletion and a extraordinary predisposition to develop cancer. It has been reported that the depletion of hematopoietic precursors is due to their high predisposition to enter into apoptosis in response to cytotoxic cytokines or environmental stresses. However, much less attention has been put in the study of the susceptibility of FA patients to develop cancer. Several groups, including ours, have reported a role of FANCA proteins apart from the one they develop in the cellular nucleus. It is now clear that at least some FANCA proteins play a role in the cytoplasm, in pathways involved in the response to cytokines and the development of a response to insults against the DNA or the cell homeostasis [2, 6, 7]. In this article we describe the overproduction of IL-1 β in FA-A patients by a mechanism involving the PI3K-AKT signaling pathway. The levels of IL-1 β produced by FA-A cells are capable of inducing proliferation in cancer cells *in vitro*, thus providing support to a possible role of IL-1 β in the progression of cancer in these patients.

IL-1 β and TNF α are considered "alarm" cytokines since they are not normally expressed in health but they are rapidly induced in inflammation. In this respect, our data are consistent with previous reports describing an excessive production of TNF α by FA-A cells [10], since it is generally believed that both cytokines should be regulated in the same way. Indeed, cytokine-detection experiments carried out in our laboratory showed a great increase in both secreted cytokines, IL-1 β and TNF α , when measured in the growth medium of FA-A lymphoblast, in comparison to the secreted levels by control cells (data in this article and not shown). However, when we analyzed the mRNA levels, TNF α showed a discrete elevation with respect to control cells (data not shown), supporting a post-translational effect of FANCA deficiency in its overproduction, and explaining why we could not detect alterations in TNF α in the array analysis. On the contrary, the IL-1 β mRNA expression was strikingly elevated not only in FA lymphoblast cell lines, but also in primary T cells and the whole mononuclear fraction obtained from FA patients blood samples, suggesting a transcriptional effect of the FANCA protein defect on IL-1 β expression.

Several transcription factors have a functional response element in the promoter of the IL-1 β gene, including NF- κ B, c/EBP β , Spi1 and HSF-1 [19, 24-26]. Probably the best known of all these is NF- κ B, due the important role that this factor has on inflammation. We then wanted to test whether or not this factor was constitutively active in FA-A lymphoblasts. We used three different approaches to analyze it. Our results discard a constitutive activation of NF- κ B in FA-A lymphoblasts as responsible for IL-1 β overexpression. In this respect, it has been recently reported that NF- κ B is actually a negative regulator of IL-1 β secretion [27]. These data contrast with the observations of Briot et al., which suggest a constitutive activation of the NF- κ B pathway in FANCA deficient cells [28]. These two opposite groups of results may indicate distinct roles for individual FANCA proteins in the cytoplasm and provide a possible explanation to the variations in the phenotype of different FA complementation groups that cannot be attributable to their cooperative role in the nuclear FA-BRCA protein complex. Interestingly, the patients we analyzed from other complementation groups (FA-D2, FA-

G and FA-J) showed also very high levels of IL-1 in their blood serum. At the present time, we do not have an explanation for this observation and further analysis of a bigger group of patients would be necessary to confirm and interpret these data correctly.

Our experiments with inhibitors of different signaling pathways showed a normalizing effect of LY294002 over the elevated levels of IL-1 β protein and mRNA in FA-A cells, and indeed, we detected an accumulation of activated AKT in these cells, suggesting a role of PI3K-AKT activation in FA cell phenotype. This may also explain the lack of activation of JNK in response to DNA damage in these cells [2], since phospho-AKT is known to repress the phosphorylation of JNK by ASK1 [29]. Moreover, P-AKT activates MDM2 protein that affects the levels of p53 protein, targeting it for degradation [30]. We have previously shown the reduced p53 protein levels in FA-A lymphoblasts with no correlation to the accumulation of its mRNA [31]. All these previous observations match with our present description of a constitutive active PI3K-AKT pathway in FA-A cells.

When investigating the possible pathways connecting the constitutive active AKT to the IL-1 β overexpression in FA cells, we began to study GSK3. This kinase phosphorylates and inhibits several transcription factors, some of them known regulators of IL-1 β expression. Interestingly, the genetic disruption of the GSK3 β gene abrogates the activation of JNK, NF- κ B and ERK, but potentiates the apoptosis induced by TNF α , a hallmark of FA cells [32]. GSK3 also phosphorylates β -catenin, labeling it for degradation. β -catenin has a prominent role in certain tumors, being essential in the maintenance of the stem phenotype of the cutaneous cancer cells [33]. Interestingly, there is evidence for the role of β -catenin signaling in squamous cell carcinomas, the major solid tumor affecting FA patients [33]. All these observations pointed to GSK3 altered phosphorylation as a mediator of the FA phenotype. To the best of our knowledge, this is the first time it has been reported that the transcription of the IL-1 β gene is regulated by the PI3K-AKT-GSK3 β pathway. Several putative response elements for transcription factors known to be regulated by GSK3 β lie within the 5' regulatory region of the IL-1 β gene. We are now focusing on the identification of the transcription factor/s mediating the overexpression in FA cells. Alternatively, β -catenin may play an important role in IL-1 β overproduction in FA cells, by means of its interaction with members of the Tcf/Lef family of transcription factors. The analysis of all of the multiple possibilities was beyond the scope of the present work.

IL-1 β overexpression in FA patients could be a risk factor to develop more aggressive neoplasias. Several groups have reported the involvement of this cytokine in the carcinogenic process, promoting tumor growth, inhibiting apoptosis and diminishing the immunological response against the tumor [34]. Although this article does not show direct interaction of the FANCA protein with any member of the PI3K-AKT pathway, previous observations support an interaction between FANCA and AKT1 [35]. We are trying to confirm such an interaction in our cells and determine the influence of it in the functional activation of the PI3K-AKT pathway. In summary, the present work reports an overproduction of IL-1 β in FA-A patients and provides strong evidence of a constitutively active PI3K-AKT-GSK3 β pathway responsible for the IL-1 β gene overexpression in FA-A cells, that can also explain many other aspects of the cellular FA phenotype.

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FIGURE LEGENDS

Figure 1: IL-1 β is overexpressed in FA-A patients. A: Total mRNA obtained from the mononuclear fraction of FA-A patients blood samples was used in an expression microarray analysis. The graph represents the mean variation in the IL-1 β and caspase 1 mRNAs expression in FA versus control cells obtained from 2 different spots (IL-1 β) or 5 different spots (caspase 1). Positive values represent overexpression while negative values represent underexpression in FA samples. B: Semiquantitative RT-PCR analysis of total mRNA obtained from the mononuclear fraction of blood samples obtained from FA-A patients and healthy donors. The expression of IL-1 β in 4 FA-A patients and 3 healthy donors is shown. β 2 microglobulin (β 2 μ) expression in the same samples is shown as loading control. C: IL-1 β mRNA expression analysis in FA-A and control lymphoblast cell lines by semiquantitative RT-PCR. D: Detection of intracellular pro-IL-1 β in whole cell extracts from FA-A and control lymphoblast cell lines by western blot. E: Detection of secreted IL-1 β in cell culture of FA-A and control lymphoblasts cell lines by ELISA. F: The same ELISA analysis as in section E was used to detect the IL-1 β levels in the serum of FA patients of the indicated complementation groups. The IL-1 β levels in 8 control serum levels were analyzed in parallel.

Figure 2: The mutation in the FANCA protein is responsible of the IL-1 β overexpression in FA-A cells.

A: Analysis of IL-1 β mRNA stability in control an FA-A cell lines. Cells were treated with 5 μ g/ml Actinomycin D for the indicated times and IL-1 β mRNA levels analyzed by semiquantitative RT-PCR. β 2 microglobulin (β 2 μ) mRNA expression is shown as loading control. B: Semiquantitative RT-PCR analysis of mRNA obtained from primary FA-A or control T lymphocytes transduced with two different viral vectors for FANCA (SFA and LFA) or GFP as control. The expression of the transduced FancA gene is also verified. β 2 microglobulin (β 2 μ) mRNA expression is shown as loading control. C: Analysis of the mRNA expression in one control and two FA-A lymphoblast cell lines. FA-A line 2 is either transduced with a empty viral vector (IEG) or with a expression vector for FancA (FA IEG). The expression of IL-1 β and FancA is shown. β 2 microglobulin (β 2 μ) expression analysis is shown as a loading control.

Figure 3: NF- κ B is not overactivated in FA-A cells. A: Semiquantitative RT-PCR analysis of different known NF- κ B target genes in FA-A and control primary T lymphocytes. β 2 microglobulin (β 2 μ) expression is shown as loading control. B: Analysis of the NF- κ B binding to the NF- κ B response element in the IL-1 β promoter by EMSA. The binding activity in nuclear protein extracts from 3 different FA-A lymphoblast cell lines is compared to 3 control lymphoblast cell lines. The right panel analyzes the specificity of the two complexes indicated by arrows through competition with the non-labelled probe (cold probe) and disruption of the upper complex with a p65 antibody. C: The mRNA expression of IL-1 β in two different FA-A lymphoblast cell lines is compared to the expression of two control cell lines after treatment with an inhibitor of the NF- κ B activation (BAY 11-7082, 5 μ M) or an inhibitor of PI3K (LY 294002, 20 μ M) by

semiquantitative RT-PCR analysis. $\beta 2$ microglobulin ($\beta 2\mu$) expression is shown as loading control.

Figure 4: The PI3K-AKT pathway is constitutively active in FA-A cells. A: Western blot analysis of whole cell extracts obtained from control and FA-A lymphoblast cell lines cultured in the presence or absence of $20\mu\text{M}$ of the PI3K inhibitor LY294002. The activation of AKT is studied by detecting Ser 473-phosphorylated AKT (P-AKT) with a specific antibody. Total AKT and alpha-tubulin protein detection are also shown as loading controls. B. Western blot analysis of whole cell extracts from control and FA-A lymphoblast cell lines transduced either with a control viral vector (IEG) or one expressing the cDNA for FancA (FA IEG). The membrane was sequentially incubated with antibodies against Ser 473-phosphorylated AKT (P-AKT), total AKT (AKT) and alpha-tubulin as loading control. C: mRNA expression analysis of IL-1 β by semiquantitative RT-PCR in FA-A and control lymphoblast cell lines treated with the specified concentrations of LY294002 (LY). $\beta 2$ microglobulin ($\beta 2\mu$) expression is analyzed as loading control. D: The secreted IL-1 β levels present in the culture medium of the same lymphoblasts analyzed in section C were quantified by ELISA.

Figure 5: GSK3 β is inhibited by AKT1 phosphorylation in FA-A cells and controls IL-1 β promoter activity. A: Western blot analysis of whole cell extracts from FA-A lymphoblasts cultured in the presence or absence of 20mM LiCl (Li+). Analysis of the serine 9 phosphorylation of GSK3 and the whole levels of β -catenin are shown. Total GSK3 and alpha-tubulin protein detection are shown as loading controls. B: Semiquantitative RT-PCR analysis of total RNA obtained from FA-A and control lymphoblasts cultured in the presence of 5 or 20mM LiCl (Li+). The expression of β -2 microglobulin ($\beta 2\mu$) as loading control and IL-1 β are analyzed. C: Expression of endogenous IL-1 β in control lymphoblasts stably transfected with a plasmid coding for GSK3 β -K85A or the empty vector as detected by semiquantitative RT-PCR. D: Protein extracts from the same cells as in panel C were analyzed with an anti-HA antibody to demonstrate the correct expression of the dominant negative construct HA-GSK3 β -K85A. E: Luciferase assay of HCT116 cells transfected with serial deletions of the IL-1 β regulatory region and grown for 24 hours in the presence of 20mM LiCl (solid bars) or 20mM NaCl as control (empty bars).

Figure 6: The IL-1 β secreted by FA-A lymphoblasts is functionally active. A: EMSA of nuclear protein extracts obtained from primary glioblastoma progenitor cells cocultured with FA-A or control lymphoblasts, using a response element for NF- κ B as a probe (left panel, lanes 1 and 2). The involvement of IL-1 β in the process is demonstrated by adding the IL-1 receptor antagonist IL-1Ra to the coculture medium (lane 3). The complexes formed after treatment with 20ng/ml of IL-1 β (right panel) are analyzed by supershift with antibodies against the p65 or p50 components of the NF- κ B factor. B: Cell cycle analysis of primary glioblastoma stem cells cocultured with FA-A lymphoblasts in the presence or absence of the IL-1 β natural specific competitor, IL-1Ra

(75µg/ml). As a control, the cell cycle analysis of the same primary glioblastoma stem cells cultured alone in the presence of IL-1β (20ng/ml) is shown. The percentage of cells in G2/M phase is displayed for every panel. C: Percentage of cells entering mitosis from a representative cell cycle analysis of HL60 cells cocultured with FA-A lymphoblasts in the presence (solid bars) or absence (empty bars) of 75µg/ml IL-1Ra. D: A representative analysis of the cells in G2/M phase in EUFA 1365 oral squamous carcinoma cell line cocultured with FA-A lymphoblast. The percentage of EUFA 1365 cells in G2/M phase after treatment with 20ng/ml IL-1β is displayed as control.

FIGURES

Figure 1

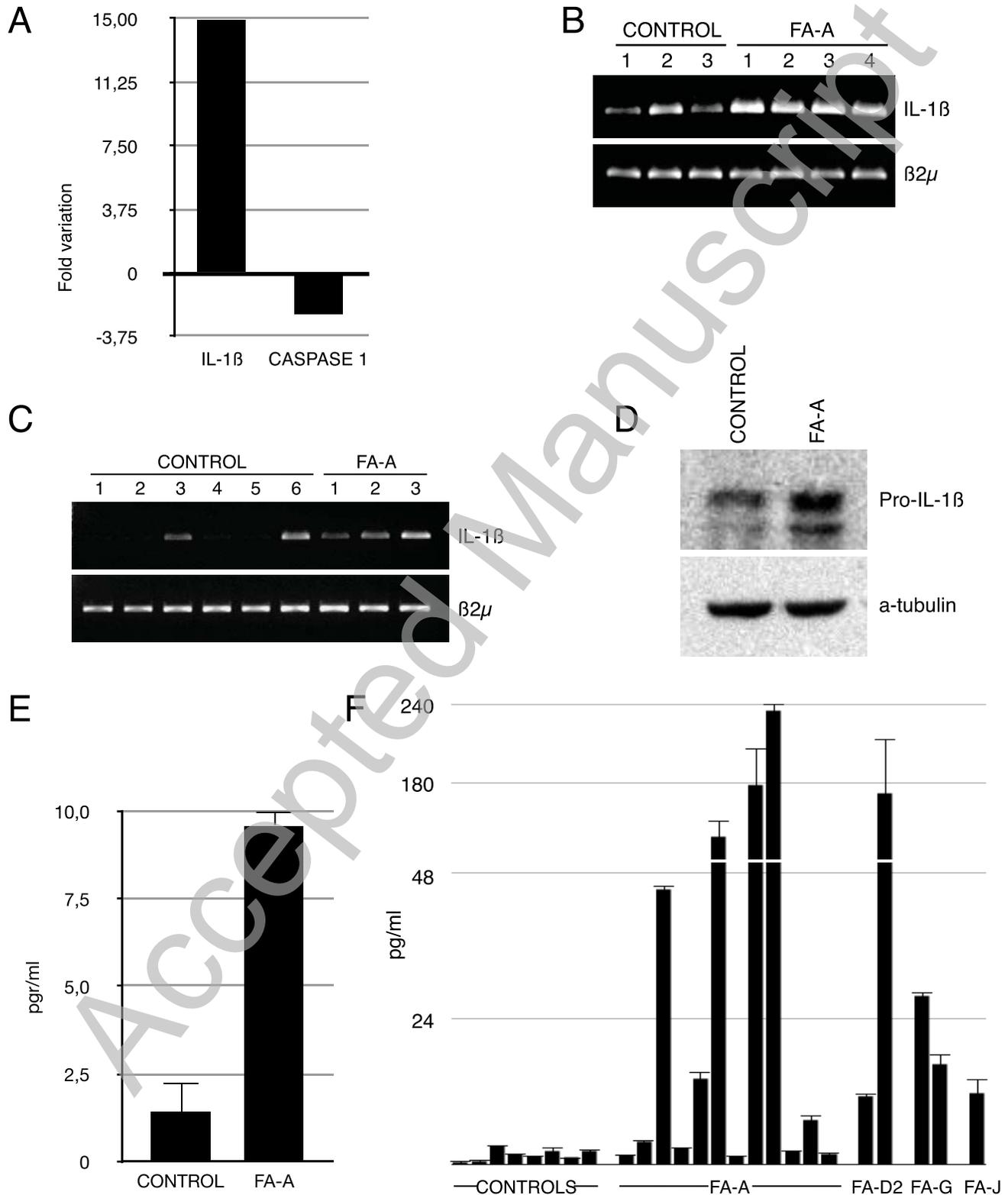
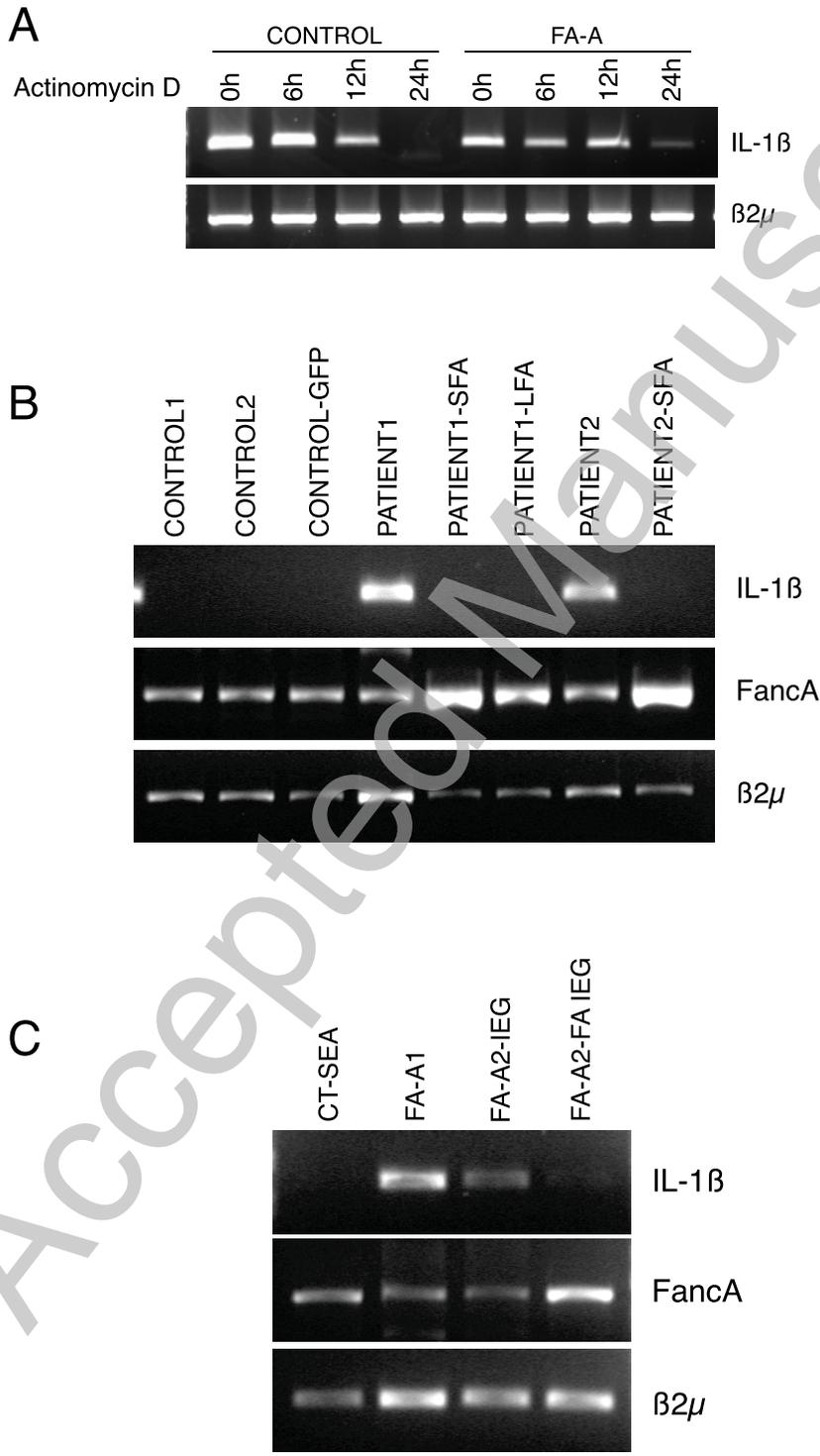


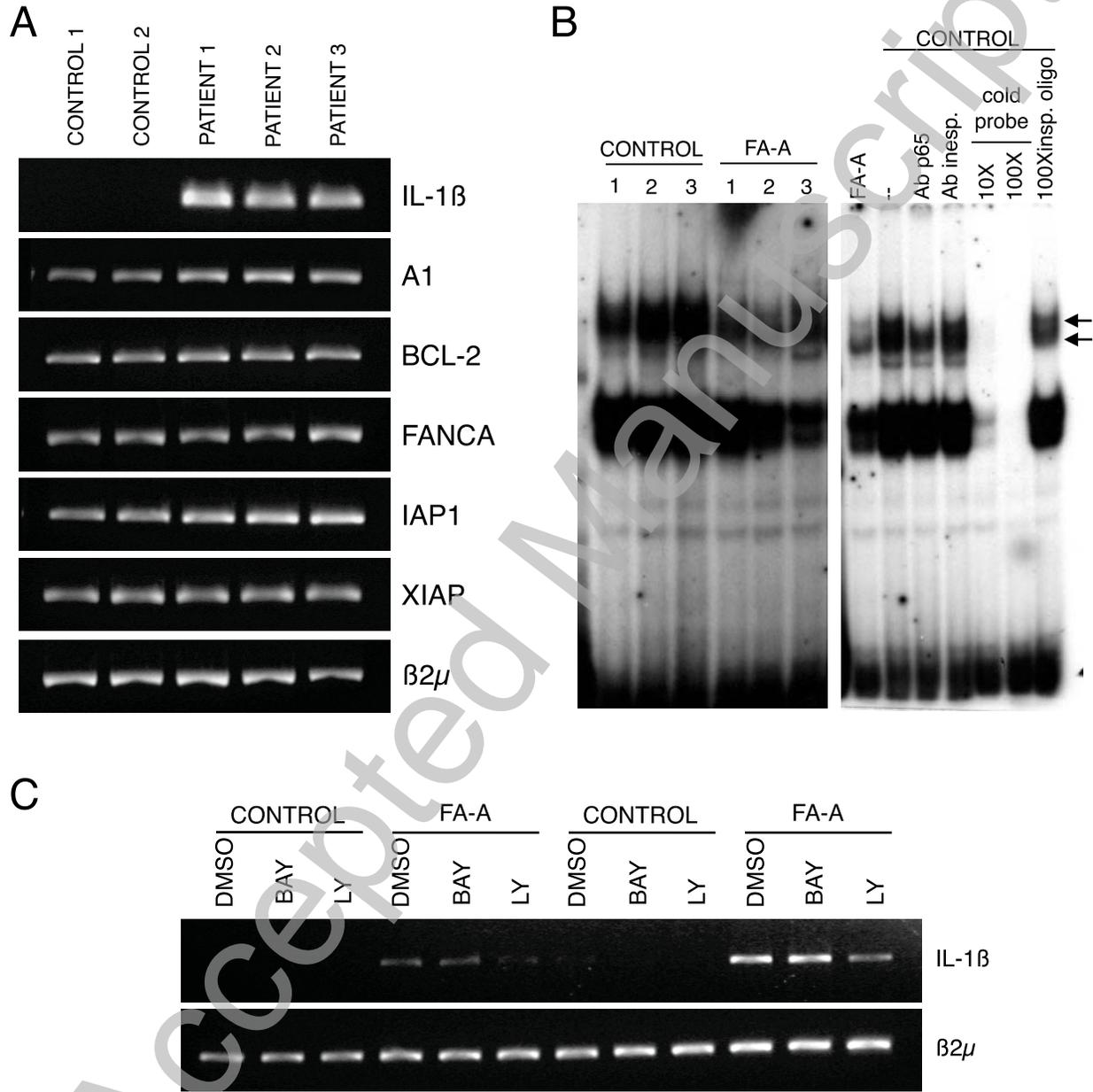
Figure 2



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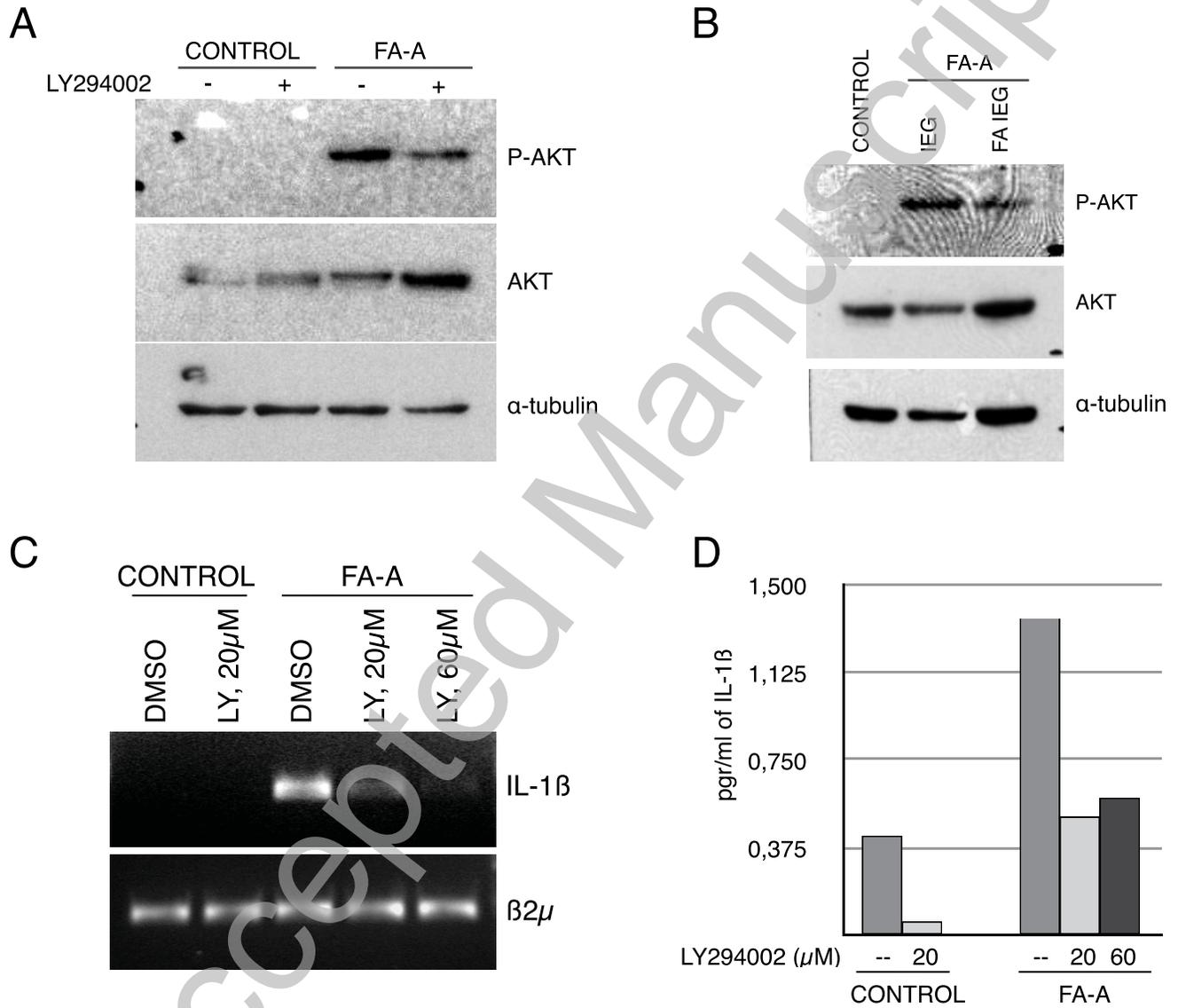
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Figure 3



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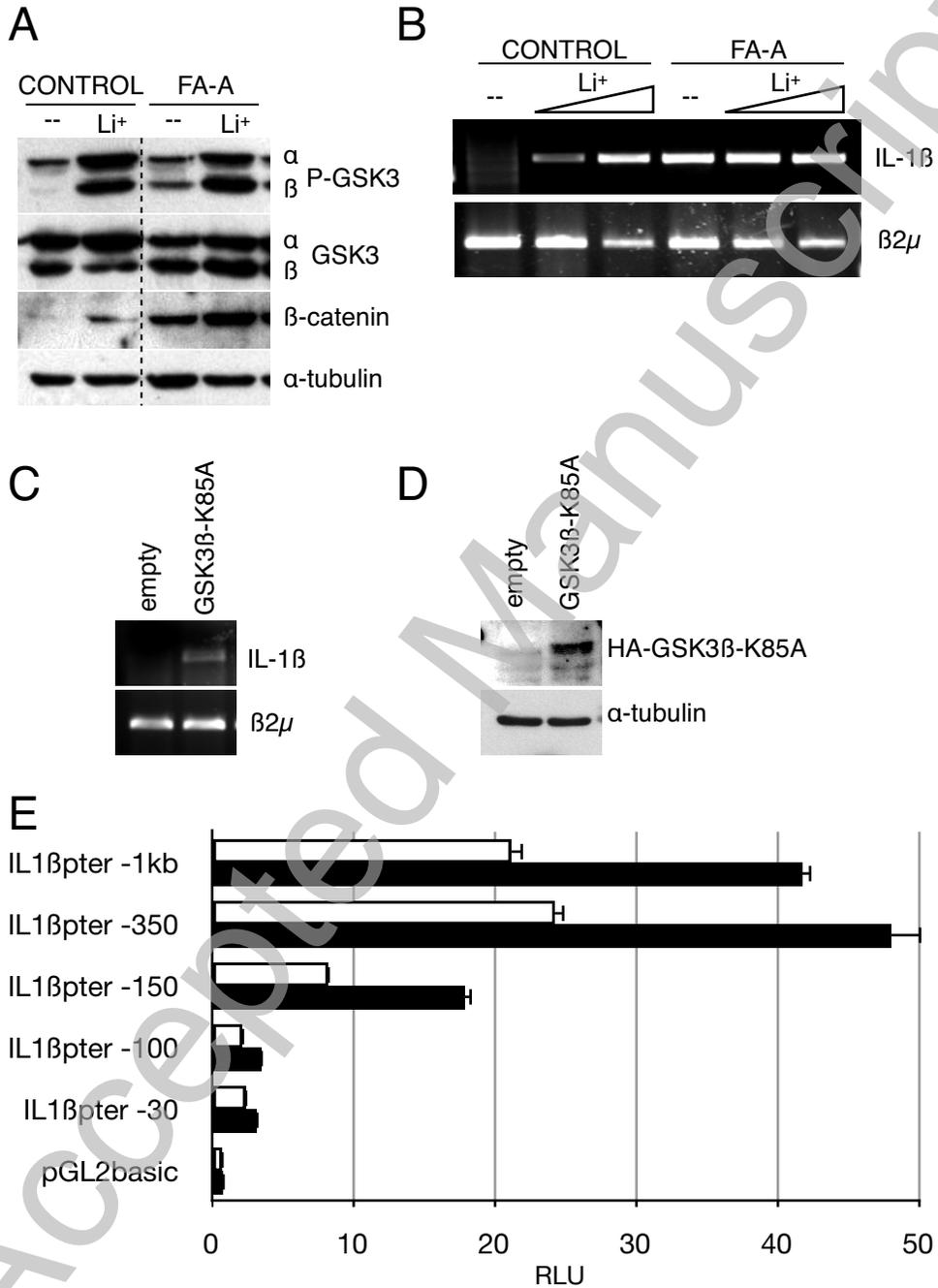
Figure 4



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Figure 5



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Figure 6

