

Overexpression of the Fanconi Anemia A Gene in Hela and MCF10A Cells

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Background: Fanconi Anemia (FA) is an autosomal recessive inherited disease, which is characterized by developmental abnormalities, progressive bone marrow failure and a predisposition to cancer. The phenotypes of FA cells show extreme sensitivities towards oxygen and DNA cross linking agents, such as diepoxybutane and mitomycin C (MMC).

Methods: In the current study, retroviruses expressing the FANCA gene were prepared to create the stable cell lines, Hela (cervical carcinoma) and MCF10A (breast). The expression of FANCA protein in the Hela and MCF10A stable cells, following puromycin selection, was checked using Western blot. The difference in the cell growth between the parent and FANCA expressing cells following MMC treatment was checked using the MTT assay.

Results: The expression of exogenous FANCA protein in the Hela and MCF10A stable cells was observed using Western blot. The MCF10A cells expressing exogenous FANCA were resistant to MMC concentrations with the range 0.01~1 μ M compared with the MCF10 parent cells. However, at an MMC concentration of 10 μ M, there was no difference in the susceptibility between the parent and FANCA expressing MCF10 cells. The Hela cells expressing FANCA showed no resistance at any MMC concentration (0.01~10 μ M).

Conclusion: FANCA protein is an important factor for resistance to the cross linking agent, MMC, in MCF10A breast cells, but not in Hela cervical carcinoma cells. (*Korean J Hematol* 2006;41:1-7.)

Key Words: Fanconi anemia, FANCA, Hela cell, MCF10A cell

INTRODUCTION

Fanconi Anemia (FA) is an autosomal recessive disorder characterized by developmental abnormalities and progressive bone marrow failure, and the incidences of aplastic anemia, myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) are all greatly increased in homo-

zygotes.¹⁻³⁾ Cells from FA patients exhibit hypersensitivity to DNA crosslinking agents, such as mitomycin C (MMC), DEB, and cisplatin.^{2,4)} Currently, more than eleven FA complementation groups are known to exist, and the nine have been identified and cloned, referred to as FA subtypes A, C, BRCA2/D1, D2, E, F, G, L and B.⁵⁻⁷⁾ At present, most (60~80%) of FA patients fall into group A. The protein of FANCA (165

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kDa) is the largest protein among other FA proteins, suggesting that FANCA gene plays an important role in FA pathogenesis. The encoded FA proteins cooperate in a common pathway: the FA/BRCA pathway.³⁾ Six of the FA proteins, A, C, E, F, G, B and L, form a protein complex constitutively found in the nucleus of cells, the FA protein complex.^{3,6-8)} The principal function of the FA complex is to activate FANCD2 following response to DNA damage.⁹⁾ The activation of FANCD2 appears to occur by the FANCL subunit of the FANCD2 complex monoubiquitinating FANCD2 on lysine 516.⁶⁾ The activated FANCD2 protein is subsequently targeted to sub-nuclear foci, which are thought to be the sites of DNA repair, and which contain BRCA1, FANCD2/BRCA2, and Rad51.^{3,10,11)}

Despite these remarkable advances, little is known about the regulation and functional role of FA proteins, especially FANCA and FANCD2. A number of recent findings have suggested that FA cells are defective in the repair of DNA double-strand breaks.¹²⁻¹⁴⁾ Repair of double-strand DNA breaks can occur by non-homologous end joining (NHEJ) or by homologous recombination (HR).^{15,16)} The repair of interstrand cross links by NHEJ, however, would be error-prone, suggesting that HR is important for the repair of that class of lesion. It is currently unclear which pathway, NHEJ or HR, is important in the FA phenotype.

To know the function of FANCA in the cancer cell lines, I expressed full length FANCA in the MCF10A breast cell and HeLa cervical carcinoma cells. I found that the MCF10A cells which expressed exogenous FANCA were resistant to the MMC, compared with the MCF10A parent cells. But HeLa cells which expressed FANCA did not show the resistance to the MMC.

MATERIALS AND METHODS

1. Cell cultures

MCF10A cells were grown in Dulbecco's modified Eagle's media-F12 (DMEM/F12, GIBCO-BRL), which

was supplemented with 5% fetal calf serum (GIBCO-BRL), penicillin (10units/mL, Sigma), streptomycin (0.1mg/mL, Sigma), 10ul EGF (1mg/mL), 5ml sodium pyruvate (100mM), 5mL glutamine (200mM), 70uL hydrocortizone (10mM), 500uL insulin (1mg/mL) and 50uL cholera toxin (1mg/mL). HeLa and 293T cells were grown in Dulbecco's modified Eagle's Medium (DMEM), which was supplemented with 10% fetal calf serum (GIBCO-BRL), penicillin (10units/mL, Sigma) and streptomycin (0.1mg/mL, Sigma).

2. Gene construct (plasmid)

The cDNA for human FANCA was from the D'Andrea lab (Dana-Farber Cancer institute, Harvard Medical School). I subcloned the cDNA of FANCA in pcDNA3 vector into the pBabe retrovirus vector by PCR using the forward primer 5'-ATCGGGGGGCCGCCAAATGTCCGACTC GTGGGTC-3' and the reverse primer 5'-ATCGGGGGGCGCGCTCAGAAGAGATGAGGCTCC-3'. The forward primer contains the FseI restriction endonuclease site and the reverse primer contains the AscI restriction endonuclease site. The pbabe puro vector contains a ZZ domain which shows the high affinity to IgG immunoglobulin. For purification of amino acids 1-232 of human FANCA, I subcloned the cDNA of FANCA in pcDNA3 vector in the pET-21a(+) with a hexhistidine tag (Novagen), by PCR using the forward primer 5'-ATCGGGGGGATCCTCCGACTCGTGGGTCCCGAACTCCGC-3' and the reverse primer 5'-ATCGGGGCTCGAGGGCCCTGGCGACGTCAGCATGCTG-3'. The forward primer contains the BamHI restriction endonuclease site and the reverse primer contains the XhoI restriction endonuclease site. The fusion protein was expressed Rosetta cells (Novagen) using standard procedures. The fusion protein was purified on Ni²⁺-NTA agarose resin (Qiagen).

3. Making the stable cell lines which express FANCA protein by retrovirus

The indicated pbabe construct was transfected

by lipofection into 293T producer cells (human embryonic kidney cells) with pKAT plasmid (packaging construct) and pHCMV-G plasmid (expressing the VSV-G envelope protein). Retroviral supernatants were collected on day 3 following lipofection. MCF10A and Hela cells were infected with the retrovirus for the 2 hour in the presence of 8 μ g/mL polybrene. Infected cells were washed free of viral supernatant and resuspended in growth media. After 2 days, cells were transferred to media containing puromycin (1 μ g/mL). Surviving cells were grown under continuous selection in puromycin.

4. Antibody and chemical

Anti-FANCA antiserum (polyclonal) was generated in rabbits using the immunogen amino acids 1-232 of human FANCA. Mitomycin C (MMC) was purchased from Sigma.

5. SDS-PAGE and Western blot analysis

Cell lysates (50 μ g per lane) were resolved on 7% SDS-polyacrylamide gels. Proteins were then transferred to polyvinylidene difluoride membrane, probed with an anti-FANCA antibody (a rabbit polyclonal IgG), followed by horseradish peroxidase-conjugated secondary antibody. Proteins were visualized by using the ECL system.

6. Growth Inhibition Assay

In vitro growth inhibition effect of MMC on MCF10A and Hela cells was determined by measuring MTT dye absorbance of living cells. Briefly, cells (2×10^5 cells/well) were seeded in 96-well microtiter plates (Nunc, Roskilde, Denmark). After exposure to the drug for 72h, 50 μ L of MTT (Sigma) solution (2mg/mL in PBS) were added to each well, and the plates were incubated for additional 4h at 37°C. MTT solution in medium was aspirated off. To achieve solubilization of the formazan crystal formed in viable cells, 200 μ L of DMSO were added to each well before absorbance at 570nm was measured.

RESULT

As shown in Fig. 1A, the human recombinant FANCA protein with ZZ domain was expressed well in 293T cell after transfection of pbabe-zz-FANCA vector (retrovirus vector). But the empty vector did not show FANCA Western band. Next, I made the retrovirus which expressed the FANCA, infected the MCF10A and Hela cells with this retrovirus and finally obtained the stable MCF10A and Hela cells which expressed the zz-FANCA protein well (Fig. 1B). I could not detect the expression of endogenous FANCA in Hela and MCF10A cells (Fig. 1B).

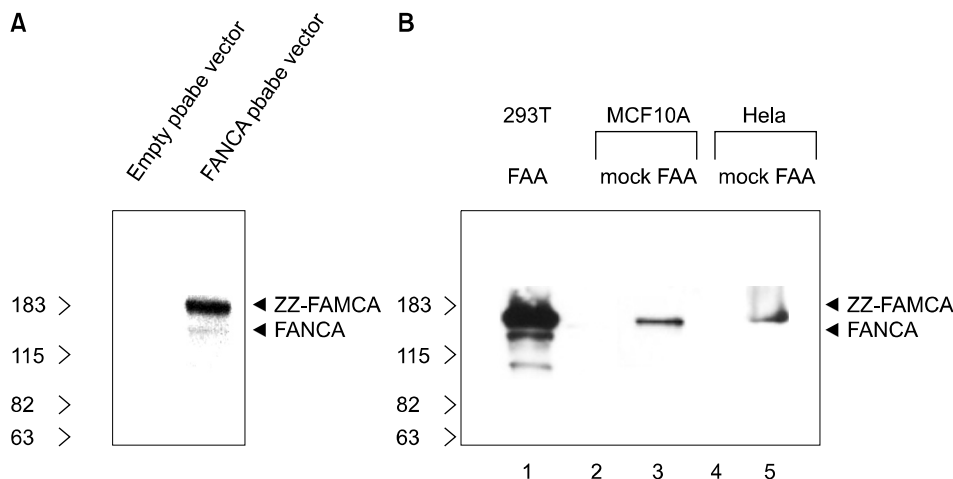


Fig. 1. FANCA expression in the stable cell lines. Western blot of the FANCA with antibodies specific for amino acids 1-232 of FANCA is shown. (A) The recombinant expression of zz-FANCA protein in 293T cells by transfection. (B) The recombinant expression of zz-FANCA protein in MCF10A breast cell and Hela cervical carcinoma cells. The mock stable cells (lanes 2 and 4) do not express the FANCA protein.

Because the exogenous level of FANCA expression is very higher than the endogenous FANCA, these stable cell lines are thought to be good for study on FANCA function only to crosslinking agents, such as MMC. Therefore, I used these cells lines for knowing whether the FANCA has a resistant function against MMC.

As shown in Fig. 2A, the FANCA stable HeLa cells did not show the resistance to MMC, com-

pared with parent cell at any doses in early and late time (24~72 hour). As for the MCF10A cell with exogenous FANCA protein, the FANCA showed a key function for resistance to MMC in the MCF10A breast cells (Fig. 2B). At the 24 hours, MCF10A cell with exogenous FANCA showed the difference of susceptibility to MMC at the lower doses (from 0.01uM and 1uM), compared with parent cells. But at the high dose (10

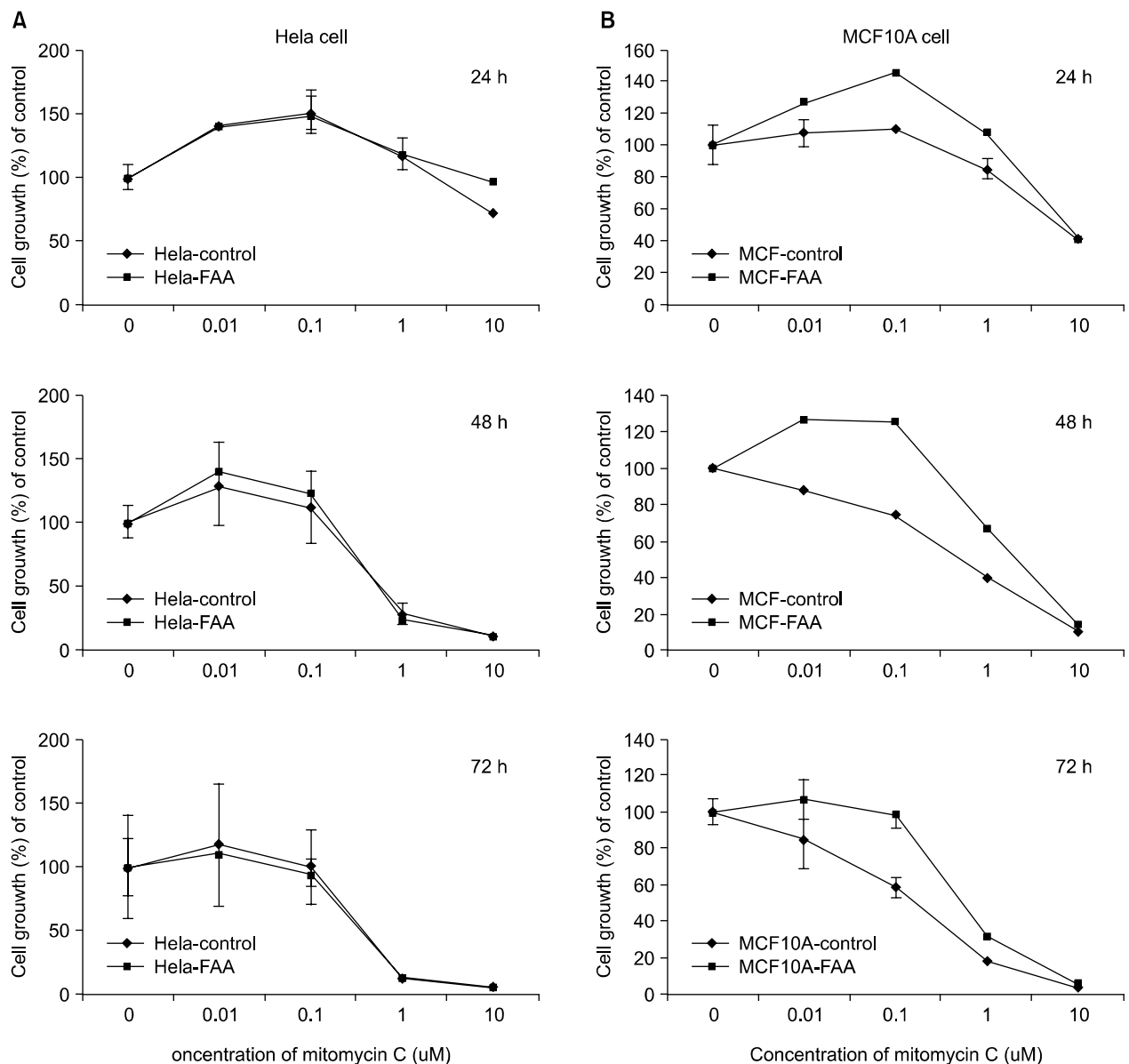


Fig. 2. (A) Effect of mitomycin C on the growth of normal HeLa cells and FANCA stable HeLa cells *in vitro*. (B) Effect of mitomycin C on the growth inhibition of normal MCF10A cells and FANCA stable MCF10A cells *in vitro*. Exponentially growing cells were treated with the indicated concentration of mitomycin C for 24, 48 and 72 h. Cell growth inhibition was assessed by MTT assay as described in "Materials and Methods". Results represent the mean of at least four independent experiments. bars, SE.

uM), the difference of resistance to MMC between the parent cells and FANCA stable cells was very low. This result indicates that the FANCA protein is very closely involved in the susceptibility to low doses of MMC (from 0.01 uM to 1uM) in the MCF10A breast cell.

DISCUSSION

Fanconi Anemia (FA) is an autosomal recessive disorder characterized by birth defects, developing pancytopenia and increased risk of malignancy. It has been shown that BRCA1 (breast cancer tumor suppressor gene) regulates two of FA proteins, called FANCA and FANCD2, by a process called ubiquitination. However, exactly how the FA proteins and BRCA1 interact to regulate DNA damage repair remains obscure. Therefore, in this experiment, I used the breast cell line (MCF10A) in which the BRCA1 and FA proteins work together for the DNA damage repair after intercross linker agents normally, and non-breast cell line (Hela cervical carcinoma cells) as a control cell line, which is not yet known whether the BRCA1 and FA proteins play a role for DNA damage repair triggered by MMC treatment.

I used the retrovirus system to make the stable cell lines expressing the FANCA protein. As shown in Fig. 1B, I successfully could detect the expression of exogenous FANCA in Hela and MCF10A cells. But I could not detect the expression of endogenous FANCA in Hela and MCF10A cells (Fig. 1B). Because I didn't use the RT-PCR to see the minimal expression of this protein, I could not exclude the possibility that these cells have the endogenous FANCA protein very rare. However, because the exogenous level of FANCA expression is very higher than the endogenous FANCA, these stable cell lines are thought to be good for study on FANCA function to crosslinking agents, such as MMC. Therefore, I used these cells lines for knowing whether the FANCA has a resistant function against MMC.

As shown in Fig. 2A, the FANCA stable Hela cells did not show the resistance to MMC, compared with parent cell at any doses in early and late time (24~72 hour). This result suggests that FANCA expression in Hela cervical carcinoma cells is not sufficient to confer resistance to MMC and Hela cells may have low expression of other FA pathway proteins such as FANCD2, FANCE, FANCL for the resistant to MMC. To be resistant to intercross linker agents such as MMC, the Hela cervical carcinoma needs more other proteins for DNA damage repair with BRCA1 than just overexpression of FANCA protein in our model.

As for the MCF10A cell with exogenous FANCA protein, the FANCA showed a key function for resistance to MMC in the MCF10A breast cells (Fig. 2B). This result indicates that the FANCA protein is very closely involved in working with BRCA1 protein as well as the other FA proteins for DNA damage repair after the MMC treatment. But we need to clarify the direct or indirect interaction of FANCA and BRCA1 in this cell by ways of immunoprecipitation and Western method or other methods. It is of interest that the only breast cell having the exogenous FANCA can show the resistance to MMC, even though I need to use more FANCA stable cell lines.

FANCA as well as FANCD2 has been implicated as an important factor in the process of DNA damage repair in response to the MMC crosslink agents and the activated FANCD2 is required for DNA damage repair with BRCA1 and BRCA2.^{1-3,10,11)} FANCA is directly bound to BRCA^{1,17)} BRCA2/FANCD1 has a well-defined role in homology-directed DNA repair.^{10,14,18-20)}

These facts suggested that FANCA and FANCD2 are tightly related to the BRCA1 and BRCA2 (breast tumor suppressor genes) and the FA pathway and BRCA pathway in cells is an important shared signaling pathways. Also, the BRCA proteins have an important role in breast cells. These reports might explain the difference of sus-

ceptibility toward MMC between the Hela cervical carcinoma cells and MCF10A breast cells, which express the exogenous FANCA protein. Even though the role of FANCA is not clear now, this protein plays more important role in breast cell rather than other tissue cells including the cervical carcinoma cells. Probably, overexpressed FANCA protein can specifically interact with BRCA1 protein in the MCF10A breast cells, resulting in more resistant to MMC. The interaction of FANCA proteins and BRCA proteins in breast cell and other cells after the intercross linker agent treatment or endogenous DNA damage will be studied further in view of the regulation of protein function by each other.

Taken together, I conclude that FANCA protein is an important factor for resistance to MMC in the MCF10A breast cell, but not the Hela cervical carcinoma cells.

요 약

배경: 판코 빈혈(Fanconi anemia)은 열성으로 유전되는 질환으로, 임상적인 특징으로는 성장에 장애가 있으며, 진행성 골수 이상과 암으로 대부분 사망하는 질환으로 알려져 있다. 특히 환자 세포에서 나타나는 주요 특징은 DNA crosslink인 mitomycin C (MMC)나 diepoxybutane에 대해 민감하게 반응을 한다.

방법: 본 실험을 하기 위해서 Hela 세포주와 MCF10A 세포주를 사용하였으며, FANCA 단백을 발현하는 안정화 세포주를 만들기 위해서 retrovirus을 이용하였다. FANCA 단백을 발현하는 세포주의 선택을 위해서 puromycin을 사용하였으며, MMC에 대한 세포주들의 성장 억제 변화를 보기 위해서 MTT assay를 이용하였다.

결과: 성공적으로 FANCA 단백을 발현하는 HeLa 세포주와 MCF10A 세포주를 얻었으며, 이들 세포에 MMC를 처리하여 세포의 성장 억제를 분석한 결과, MMC 농도에 따라(0.01~10uM) HeLa 세포주와 FANCA를 발현시킨 HeLa 세포주는 세포성장의 차이에 아무런 변화를 보이지 않았다. MCF10A 세포주는 MMC 농도 0.01uM 부터 성적 억제가 관찰되었고(72 시간 배양), FANCA를 발현시킨 MCF10A 세포주는 MMC 농도(0.01~0.1uM)에서 성장이 확연히 덜 억제됨을 관찰

할 수 있었다.

결론: FANCA 단백질은 breast 세포주인 MCF10A에서 MMC에 대한 감수성을 억제하는데 중요하게 작용하는 하나의 인자임을 확인하였다.

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