Title Page

[Original Article]

Phosphorylation status of Fas-associated death domain protein is associated with biochemical recurrence after radical prostatectomy

Tomohiro Ikeda[1], Nobumichi Tanaka[1], Keiji Shimada[2], Yoshiaki Matsumura[1], Makito Miyake[1], Satoshi Anai[1], Atsushi Tomioka[1], Eijiro Okajima[1], Akihide Hirayama[1], Kiyohide Fujimoto[1], Noboru Konishi[2], Yoshihiko Hirao[1]

1. Department of Urology, Nara Medical University, Kashihara, Nara, Japan

2. Department of Pathology, Nara Medical University, Kashihara, Nara, Japan,

***Correspondence should be addressed to:** Nobumichi Tanaka, M.D.

Department of Urology, Nara Medical University

840 Shijo-cho, Kashihara, Nara 634-8522, Japan.

Tel: +81 744 22 3051 (ext. 2338)

Fax: +81 744 22 9282

E-mail: sendo@naramed-u.ac.jp

Key words: Prostate cancer, radical prostatectomy, prognostic factor, biochemical recurrence, p-FADD

Abstract

Objectives

To assess whether phosphorylated FADD at 194 serine (p-FADD) is valuable as a marker of biochemical recurrence in hormone-naïve patients who had undergone radical prostatectomy. Fas-associated death domain protein (FADD) is one of the death receptor family members, and is well known to be associated with the execution of Fas-mediated apoptosis in various types of cancer cells. We have previously demonstrated that p-FADD is associated with prostate cancer progression, and that a positive p-FADD could be a useful marker to predict biochemical recurrence after radical prostatectomy (RP) in patients undergoing neoadjuvant androgen deprivation therapy.

Materials and Methods

We used radical prostatectomy specimens from 106 patients. None of the patients had received neoadjuvant or adjuvant therapy. The percentage of positive p-FADD cells (nuclear staining) was immunohistochemically evaluated. The correlation between FADD phosphorylation and clinicopathological parameters was assessed. The correlation between the biochemical recurrence-free rate and the p-FADD expression level was analyzed by the Kaplan-Meier method.

Results

Overall, 39 patients showed biochemical recurrence. We investigated the expression of p-FADD in 106 prostate cancer patients using immunohistochemistry, and we compared our findings with clinicopathological parameters including follow-up data. Patients with a

higher positive p-FADD rate showed a significantly lower biochemical recurrence rate than those with a lower positive p-FADD rate ($p<0.001$). There was a significant inverse correlation between the positive p-FADD rate and the Gleason score.

Conclusion

Low expression of p-FADD could be a predictor of biochemical recurrence in hormone-naïve patients who had undergone radical prostatectomy.

Manuscript

Introduction

Prostate cancer is a common cancer in men, and its incidence has been increasing in Japan. Although most prostate cancer patients have a relatively good prognosis after primary treatment, some of the patients have a poor prognosis. The prognosis of patients who had undergone radical prostatectomy (RP) is uncertain, even if the Gleason score is used. Organ-confined prostate cancer can be curatively treated with surgery. However, once metastasis occurs, the disease will eventually take a fatal course. This fact underscores the necessity of novel prognostic markers. We then focused on the phosphorylation status of FADD in prostatectomy specimens.

Fas-associated death domain protein (FADD) is one of the death receptor family members, and is well known to be associated with the execution of Fas-mediated apoptosis in various types of cancer cells (1-3). It has previously been reported that FADD was phosphorylated at serine194 during the G2/M period, and phosphorylated FADD is closely associated with cell cycle regulation (4). We have demonstrated that phosphorylated FADD at 194 serine (p-FADD) is associated with prostate cancer progression, and showed a correlation between the Gleason score and a positive rate of p-FADD in prostate cancer specimens (5). In addition, we reported a correlation between the biochemical recurrence rate and a positive rate of p-FADD staining in patients who had undergone RP following neoadjuvant hormonal treatment. According to our data, with a

p-FADD cut-off value of 15%, the group with more than 15% showed a significantly higher biochemical recurrence-free rate (6).

In this study, we assessed whether p-FADD is a potential marker to predict biochemical recurrence in hormone-naïve patients who underwent RP.

Materials and methods

Tissue samples and patients

In this study we examined 106 primary tissue specimens that were obtained from hormone-naïve patients who underwent RP from 1999 to 2006 at our hospital. Patients' age ranged from 53 to 78 (median 68.4). Biochemical recurrence was defined as a PSA level of 0.2 ng/mL or higher.

The median prostate-specific antigen (PSA) level of the prostatic biopsy was 11.8 ng/mL (range 3.9 to 48.0 ng/mL; Tandem R assay). We divided the patients into two groups, those with and those without recurrence. The median follow-up period was 36.4 months (range: 14 – 85 months). Patients' characteristics are shown in Table 1, 2. There were no significant differences between the recurrence-free group and the recurrence group, except for nadir PSA and clinical stage.

Immunohistochemistry

Sections of the specimens were incubated for 16 h at 4°C and reactions were visualized using a Histofine SAB-PO kit and diaminobenzidine as the chromogen (Nichirei, Tokyo, Japan), with haematoxylin counterstaining.

The antibody used in the experiment was polyclonal antibody(CST company). It was diluted 50 times with PBS. We assessed the percentage of positive nuclear staining for p-FADD (p-FADD (%)). The number of phospho-FADD-positive cells per 100 cells was designated as the percentage of positive cells in at least 1000 examined cells. The image of immunohistochemical staining of p-FADD is shown in Fig.1. We immunohistochemically examined the correlation between FADD phosphorylation in RP specimens and clinicopathological parameters in patients who underwent RP and the biochemical recurrence-free rate stratified by the expression levels of p-FADD in RP specimens. We decided on a p-FADD cut-off value of 15% for analysis of the area under the curve of the ROC curve. The cut-off value of p-FADD expression was defined as 15%, which was the highest sum of both sensitivity (86.6%) and specificity (82.1%).

Statistical analysis

Wilcoxon's signed rank test was used to analyze the distribution of the percentage of p-FADD positive cells in relation to morphology. Statistical analyses for intergroup comparison were carried out using the Mann-Whitney U test and Fisher's exact probability test. Survival analysis of biochemical recurrence was calculated by the Kaplan-Meier method and the log-rank test. All statistical tests were two-sided, and statistical significance was defined as $p<0.05$.

Informed consent was obtained from all patients before specimens were collected, as appropriate. The study was approved by the Ethics Committee

of Nara Medical University.

Results

Expression of phosphorylated FADD at serine 194 in prostate tissue

We investigated the phosphorylation status of FADD in normal epithelial cells and prostate carcinoma specimens immunohistochemically (Fig.1). Expression of the serine 194-phosphorylated form of FADD was significantly higher in normal prostate epithelial cells, where it was predominantly located in the nucleus, but it was lower in cancer cells. Phosphorylated FADD at 194 serine was also more expressed in cancer cells of patients who did not have recurrence.

Correlation between FADD phosphorylation in RP specimens and clinicopathological parameters

Of the 106 patients, 39 patients showed biochemical recurrence during the follow-up period. We compared the positive rate of FADD phosphorylation in RP specimens with clinicopathological parameters.
The positive rate of p-FADD in the recurrence group was significantly lower than in the recurrence-free group. The positive rate of p-FADD was significantly lower in the recurrence group (Fig. 2-A). The positive rate of p-FADD was significantly lower in patients with a Gleason score of 8-10,

both in biopsy and surgical specimens, whereas there were no significant differences in the PSA levels at diagnosis (Fig. 2-B).

Patients with a higher positive rate of p-FADD showed a significantly lower biochemical recurrence rate.

When the p-FADD cut-off value was defined as 15%, as in the previous study, the Kaplan-Meier analysis showed that the group with more than 15% had a significantly higher biochemical recurrence-free rate (p<0.001) (Fig.3).

Discussion

We have previously reported that phosphorylated FADD at 194 serine (p-FADD) is associated with prostate cancer progression and that there is a correlation between the biochemical recurrence rate and a positive rate of p-FADD staining in patients who underwent RP following neoadjuvant androgen deprivation therapy (6). In this study, we clarified the association between biochemical recurrence rate and the positive rate of p-FADD staining in hormone-naïve patients. Fas-associated death domain protein (FADD) is one of the death receptor family members, and is well known to be associated with the execution of Fas-mediated apoptosis in various types of cancer cells. The FADD gene is located on chromosome 11q13.3 in humans. The FADD protein consists of 208 amino acids. The death

domain (DD) and death effector domain (DED) are essential for interaction with death receptors and transmission of the apoptotic signal. Furthermore, the human Ser 194 phosphorylation site has a crucial role in survival/proliferation and cell cycle progression. FADD also plays a role in embryonic development (7). Shimada et al. reported that FADD is more dephosphorylated in prostate cancer cells than in normal epithelial cells, that the extent of FADD phosphorylation correlates inversely with the PSA level, Gleason score, and several pathological parameters of cancer invasion, and that phosphorylation of FADD alone cannot affect cytotoxicity, but that it contributes to chemosensitization in prostate cancer (5). Our previous report revealed that the phosphorylation status of FADD at S194 correlates with hTERT expression and telomerase activity, and that the phosphorylation status of FADD at S194 regulates prostate cancer proliferation, invasion and sensitivity to anti-cancer agents (6).

Our present study showed that the phosphorylated form of FADD stained in the nucleus, and that FADD stained in the cytoplasm. Overexpression of p-FADD was noted in normal prostatic epithelial cells. In prostate adenocarcinoma, the positive rates of p-FADD were lower than in normal prostatic epithelial cells. We also showed that the positive rates of p-FADD tended to decrease when the Gleason score increased. Interestingly, there was no correlation between the positive rate of p-FADD and PSA at diagnosis. This result may show that PSA does not always reflect the condition of the prostate cancer. Patients with a higher positive rate of p-FADD showed a significantly lower biochemical recurrence rate than those with lower positivity for p-FADD. We divided patients into two groups

by the level of the positive rate of p-FADD. When the p-FADD cut-off value was 15%, as in the previous study, the group with more than 15% showed a significantly higher biochemical recurrence-free rate (p<0.001). Low expression of p-FADD can be a predictive marker of biochemical recurrence in a patient who had undergone RP.

According to several previous reports, the significance of FADD as a prognostic factor is controversial. In lung adenocarcinoma, Chen et al. demonstrated that phosphorylated FADD induces NF-κB, upsets the cell cycle, and is associated with a poor outcome (8). In gastric cancer, Nam et al. demonstrated that there was not a significant association between p-FADD expression and clinicopathological characteristics, including invasion, metastasis, and stage (9).

These controversial results may reflect that FADD function is associated with both cell apoptosis and survival/proliferation, and that it is different in each organ.

Our findings may not only hint at an important role of p-FADD in prostate cancer progression, but also suggest p-FADD expression as a novel prognostic marker for prostate cancer.

Regarding the association between prostate cancer therapy and p-FADD expression, Shimada et al. showed that FADD phosphorylation at Ser194 affects the functions of the MAP kinase pathway and that it is closely associated with chemosensitivity in prostate cancer cells, and that it also plays an essential role in the mechanism of the amplification of chemotherapy-induced apoptosis (10-14). We conducted this study to confirm whether FADD phosphorylation could be a biomarker to evaluate

the clinical therapeutic outcome.

Although we believe that p-FADD expression is associated with the outcome of prostate cancer therapy, the pathogenesis of uncontrolled cells does not depend on one single key molecule, but on more than one. Many studies of immunohistochemical examination of prostate cancer and other cancers have been reported. Bauer et al. concluded that p53 and bcl-2 appear to be important biomarkers that predict recurrence in clinically localized prostate cancer after RP (15). By using these markers together, it may be possible to achieve a lower recurrence rate and to provide additional therapy at a more appropriate time, than if each marker is used individually.

References

1, Chinnaiyan,A.M., O'Rourke,K., Tewari,M. et al.: FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell 1995; 81: 505-512.

2, Boldin,M.P., Goncharov,T.M., Goltsev,Y.V. et al. : Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. Cell 1996 ; 85: 803-815.

3, Nagata,S. and Golstein,P.: The Fas death factor. Science: 1995; 267:1449-1456.

4, Scaffidi C, Volkland J, Blomberg I, et al.: Phosphorylation of FADD/MORT1 at serine 194 and association with a 70-kDa cell cycle-regulated protein kinase. J Immunol 2000; 164:1236-1242.

5, Shimada K, Matsuyoshi S, Nakamura M, et al.: Phosphorylation status of Fas-associated death domain-containing protein (FADD) is associated with prostate cancer progression. J Pathol 2005; 206: 423-432.

6, Yoshiaki M, Keiji S, Nobumichi T, et al.: Phosphorylation Status of Fas-Associated Death Domain-Containing Protein Regulates Telomerase Activity and Strongly Correlates with Prostate Cancer Outcomes. Pathobiology 2009; 76:293-302

7, Marion M, Ann W: Apoptosis and disease: a life or death decision. EMBO reports. 2004; 5, 674-678

8, Chen G, Bhojani MS, Heaford AC, et al.: Phosphorylated FADD induces NF-kappaB, perturbs cell cycle, and is associated with poor outcome in lung adenocarcinomas. *Proc Natl Acad Sci U S A*. 2005; 102: 12507-12.

9, Nam Jin Yoo, Sung Hak Lee, Sug Hyung Lee, et al.: Expression of nuclear and cytoplasmic phosphorylated FADD in gastric cancers

Pathology **203 (2007) 73-78.**

10, Keiji S, Syuichi M, Mitsutoshi N, et al.: Phosphorylation of FADD is critical for sensitivity to anticancer drug-induced apoptosis Carcinogenesis. 2004; 25; 1089-97

11, Shimada K, Nakamura M, Ishida E, et al.: Molecular roles of MAP kinases and FADD phosphorylation in prostate cancer. Histol Histopathol;2006; 21: 415-422

12, Shimada,K., Nakamura,M., Ishida,E., et al.: c-Jun NH2-terminal kinase-dependent fas activation contributes to etoposide-induced apoptosis in p53-mutated prostate cancer cells. Prostate 2003; 55: 265-280.

13, Shimada,K., Nakamura,M., Ishida,E., et al.:Phosphorylation of Fas-associated death domain contributes to enhancement of etoposide-induced apoptosis in prostate cancer cells. Jpn. J. Cancer Res.2002; 93, 1164-1174.

14, Shimada,K., Nakamura,M., Ishida,E., et al.: Contributions of mitogen-activated protein kinase and nuclear factor kappa B to N-(4-hydroxyphenyl)retinamide-induced apoptosis in prostate cancer cells. Mol. Carcinog:2002; 35, 127-137.

15, John J. Bauer, Isabella. Sesterhenn, Judd W. Moul, et al.: Elevated levels of apoptosis regulator proteins p53 and surgically treated clinically localized prostate cancer bcl-2 are independent prognostic biomarkers in surgically treated clinically localized prostate cancer. J.Urology :1996;156; 1511-6.

Table 1. Patient characteristics at diagnosis

Fig. 1

This shows an image of immunohistochemical staining of p-FADD. The nuclei are stained.

Fig.2

This graph shows the relationship between FADD phosphorylation in RP specimens and clinicopathological parameters. In the recurrence group, the positive rate of p-FADD was significantly lower. When the Gleason score of prostatectomy specimens increases, the positive rate of p-FADD decreases.

Fig.3

This graph shows the correlation between the positive rate of p-FADD and biochemical recurrence after prostatectomy. When the p-FADD cut-off value was 15% as in the previous study, the group with more than 15% showed a significantly higher biochemical recurrence-free rate.