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Research Paper

Variants of FasL and ABCC5 are predictive of outcome after chemotherapy-based treatment in osteosarcoma

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ABSTRACT

Objectives: Previous pharmacogenetics studies showed that genetic variants could be indicative of the response to chemotherapy. We aimed to investigate whether variants of FasL, MSH2, ABCC5, CASP3 and CYP3A4 are associated with the outcome after chemotherapy-based treatment in osteosarcoma.

Methods: 132 osteosarcoma patients who had completed the neoadjuvant chemotherapy in our center were included. 5-year progression-free survival (PFS) was assessed from the initial treatment to the earliest sign of disease progression or death from any cause. 5 SNPs were genotyped using TaqMan SNP Genotyping Assay, including rs763110 of FasL, rs4638843 of MSH2, rs939338 of ABCC5, rs2720376 of CASP3 and rs4646437 of CYP3A4. Patients were classified into two groups according to the 5-year PFS (event/no event). The chi-square test was used to analyze difference of genotype frequency. Logistic regression analysis was used to determine the independent predictors of the PFS rate.

Results: The overall 5-year PFS was 61.4% (81/132). Genotype TT/CT of rs763110 and genotype GG/AG of rs939338 were significantly associated with the event of 5-year PFS ($p = 0.028$ for rs763110; $p = 0.039$ for rs939338). Patients with no risk allele showed a 5-year PFS of 73.7% (42/57), which was significantly higher than a PFS of 54.2% (26/48) for patients with one risk allele and 48.1% (13/27) for patients with two different risk alleles ($p = 0.03$). Logistic regression analysis showed that allele T of FasL rs763110 and allele G of ABCC5 rs939338 were independent risk factors of the 5-year PFS. The ORs were 2.14 (95%CI = 1.13–3.35, $p = 0.01$) for rs763110 and 1.73 (95%CI = 1.05–2.52, $p = 0.03$) for rs939338, respectively.

Conclusions: The association of variants of FASL and ABCC5 with survival outcome after chemotherapy was validated in patients with osteosarcoma. Our findings may provide a new insight into a more personalized treatment for patients with osteosarcoma.

1. Introduction

Osteosarcoma is a common primary malignant bone tumor predominantly located in the metaphyses of the distal femur, proximal tibia and proximal humerus [1]. Since its advent in 1970s, neoadjuvant chemotherapy has greatly promoted the survival rate of osteosarcoma patients [2,3]. The backbone drugs currently used in neoadjuvant chemotherapy of osteosarcoma included doxorubicin, cisplatin, methotrexate and ifosfamide [4]. Despite the reported effectiveness of these chemotherapeutics, patients were found to have highly varied sensitivity to these agents regarding the antitumor effect and the toxic side-effect [5]. To date, few predictors have been reported to be indicative of the overall survival after the completion of neoadjuvant chemotherapy [6–8]. Reliable predictive factors await to be uncovered to identify patients who may benefit less from the chemotherapy.

Pharmacogenetics of cancer treatment mainly refers to the inherited variability of drug response [9]. In previous studies, candidate gene analysis and pathways-based gene analysis were employed to explore pharmacogenetic markers [5]. To date, a few genetic polymorphisms have been found predictive of sensitivity and toxicity of chemotherapy in osteosarcoma through candidate gene analysis. Polymorphisms of ERCC1 were reported to play important roles in the response to cisplatin mediated by the DNA repair pathway [10]. MTHFR C677T polymorphism was identified as a predictor of MTX toxicity in osteosarcoma patients [11]. The SNPs of ABCB1 and ABCC3 were found significantly associated with pharmacokinetic parameters and the occurrence of MTX toxicity [12,13]. To facilitate risk stratification and personalized treatment, establishment of a prediction model based on these genetic variants are warranted.

In a recent study, Hagleitner et al. [14] investigated genes variations

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involved in the metabolism of cisplatin and doxorubicin in 126 patients with osteosarcoma. Five variants in FasL, MSH2, ABCC5, CASP3 and CYP3A4 were combined in a risk prediction model to differentiate patients with different progression-free survival (PFS) [14]. The authors reported a PFS of 100% in patients with none or only one risk allele [14]. Since there exists remarkable genomic heterogeneity in osteosarcoma, replication of these five SNPs in additional cohorts of patients is warranted to confirm the efficacy of the predictive model. In the current study, we investigated the association of five variants of FasL, MSH2, ABCC5, CASP3 and CYP3A4 with the 5-year PFS in a cohort of osteosarcoma patients who had completed the neoadjuvant chemotherapy. Moreover, we analyzed the functional role of these variants in the gene expression.

2. Methods

2.1. Subjects

This study was approved by the ethics committee of our University Medical Center. 132 osteosarcoma patients who had completed the neoadjuvant chemotherapy in our center were included. Informed consent was obtained from all patients or their guardians. All the patients underwent the same chemotherapy regimens consisted of cisplatin (300 mg/m^2), doxorubicin (80 mg/m^2) and methotrexate (10 g/m^2) with a minimum of 5-year follow-up. Clinical data were collected from the medical record retrospectively. PFS was assessed from the initial treatment to the earliest sign of disease progression or death from any cause. Good response to the treatment was defined as with $<10\%$ vital cells after two cycles of preoperative chemotherapy therapy [15].

2.2. Genotyping of target variants

The peripheral blood was collected from each patient at the initial visit. DNA was then extracted using the DNA extraction kit (QIAGEN Inc., Tokyo, Japan) according to the protocol of the manufacturers. 5 SNPs were genotyped using TaqMan SNP Genotyping Assay, including rs763110 of FasL, rs4638843 of MSH2, rs939338 of ABCC5, rs2720376 of CASP3 and rs4646437 of CYP3A4 [14]. The genotyping assay was performed with ABI 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Ten percent of the samples were randomly selected to validate the genotyping results. The reproducible rate was 100%.

2.3. Expression analysis in tumor samples

The tumor samples were collected from 56 patients during the resection surgery and stored at −80 °C. The total RNA was extracted from the tumor tissue using the TaKaRa MiniBEST Universal RNA Extraction Kit (TaKaRa, Tokyo, Japan). Real-time PCR was carried out to quantify the mRNA expression level for above-mentioned 5 genes on the ABI 7900HT Detection System (Applied Biosystems, Foster City, CA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous control gene. The specific primers of the target genes were listed in Table 1. All amplification procedures were repeated in triplicate, with a mean value of threshold cycle (Ct) scores used to calculate the expression level with ΔΔCt method.

2.4. Statistical analyses

The Hardy–Weinberg equilibrium (HWE) test was performed to exclude selection bias. Patients were classified into two groups according to the 5-year PFS (event/no event). For the inter-group comparison, the Chi-square test was used to analyze difference of genotype frequency and the Student's *t* test was used to analyze the difference of gene expression level. Specifically, a dominant model was used to compare the association between genotype and PFS. The odds ratios (OR) and 95% confidence interval (95% CI) were calculated with the risk allele as reference. In addition, the student *t* test was used to compare the gene expression level between patients with risk allele and those without risk-allele. Logistic regression analysis was used to determine the independent predictors of the PFS rate. Genotype was coded as 0 for homozygotes of non-risk allele and 1 for heterozygotes and homozygotes of risk allele. Response to preoperative chemotherapy treatment was coded as 0 for good response and 1 for bad response. According to different number of the risk alleles, the 5-year PFS curves were analyzed using the Kaplan–Meier method. All the statistical analyses were performed with the SPSS software (version 17.0, Chicago, IL). Statistical significance was set at $p < 0.05$.

3. Results

3.1. Demographic data of the patients

The mean age of patients was 35.2 ± 19.8 years old. 74 patients (56.1%) had tumor metastasis at the initial visit. 102 (77.3%) patients received limb salvage surgery. 49 (37.1%) patients showed good response to chemotherapy. The overall 5-year PFS was 61.4% (81/132). By the end of the 5-year follow-up, 42 (31.8%) patients had died for tumor recurrence or metastasis.

3.2. Genetic association with 5-year PFS

The genotyping results of the 5 SNPs were listed in Table 2. Genotype TT/CT of rs763110 and genotype GG/AG of rs939338 were significantly associated with the event of 5-year PFS ($p = 0.028$ for rs763110; $p = 0.039$ for rs939338). The frequency of genotype CC of rs4638843 was 1. As for rs2720376 and rs4646437, no significant association with 5-year PFS was found. Patients with no risk allele showed a 5-year PFS of 73.7% (42/57), which was significantly higher than a PFS of 54.2% (26/48) for patients with one risk allele and 48.1% (13/27) for patients with two different risk alleles ($p = 0.03$, Fig. 1).

3.3. Logistic regression analysis of risk factors

Logistic regression analysis showed that allele T of FasL rs763110 and allele G of ABCC5 rs939338 were independent risk factors of the 5-year PFS. The ORs were 2.14 (95%CI = 1.13–3.35, $p = 0.01$) for rs763110 and 1.73 (95%CI = 1.05–2.52, $p = 0.03$) for rs939338, respectively. With the cut-off value set as 0.5, the sensitivity and

Table 1
Primers for the qPCR assay.

Gene	Forward primer	Reverse primer
FasL	TGCCTTGGTAGGATTGGGC	GCTGGTAGACTCTCGGAGTTC
ABCC5	TGTTTTGCTGCAGGGCTCA	AGTGCTGGTTCTCTCCCTCA
MSH2	**AGGCATCCAAGGAGAATGATTG**	**GGAATCCACATACCCAACTCCAA**
CASP3	**CATGGAAGCGAATCAATGGACT**	**CTGTACCAGACCGAGATGTCA**
CYP3A4	**AGATGCCTTTAGGTCCAATGGG**	**GCTGGAGATAGCAATGTTCGT**
GAPDH	GAGTCAACGGATTTGGTCGT	TTGATTTTGGAGGGATCTCG

Table 2
Association of the four SNPs with 5-year PFS.

SNPs	Genotype[a]		Allele type		*p*		Odds ratio (95%CI)[b]
	Event (n = 51)	No event (n = 81)	Event	No event	Genotype	Allele	
rs763110 (T/C)	27/24	26/55	52/50	51/111	0.028	0.002	2.26 (1.35–3.77)
rs939338 (G/A)	25/26	24/57	46/56	47/115	0.039	0.008	2.01 (1.20–3.37)
rs2720376 (T/C)	19/32	28/53	35/67	55/107	0.89	0.95	1.02 (0.62–1.71)
rs4646437 (A/G)	11/40	23/58	21/81	44/118	0.51	0.24	0.69 (0.39–1.26)

[a] The two values in the 'genotype' column indicate the number of homozygotes with respect to the mutant allele plus heterozygotes and the number of homozygotes with respect to the wildtype allele, respectively. i.e. for rs763110, the two values indicate the number of genotype TT + TC and the number of genotype CC, respectively.
[b] Calculated for the alleles.

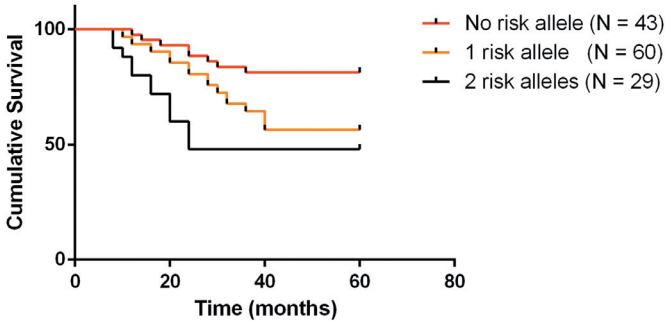

Fig. 1. Five-year PFS based on number of risk allele.
Patients with no risk allele showed a 5-year PFS of 81.4%, which was significantly higher than a PFS of 55.0% for patients with one risk allele and 44.8% for patients with two different risk alleles (p = 0.003).

specificity of the regression model were 81.5% and 63.3%, respectively. No significant association was found between 5-year PFS and other factors including rs4646437 of CYP3A4, rs2720376 of CASP3 and response to the chemotherapy.

3.4. Relationship between gene expression and 5-year PFS

Of the 56 patients, 4 patients were excluded from the expression analysis due to the degradation of the tissue. As shown in Table 3, patients with no event of PFS were found to have significantly higher expression of FasL and lower expression of ABCC5 than those with event of PFS (0.00037 ± 0.00021 vs. 0.00025 ± 0.00014, p = 0.02 for FasL; 0.0058 ± 0.0027 vs. 0.0084 ± 0.0041, p = 0.008 for ABCC5). As for MSH2, CASP3 and CYP3A4, no significant difference was found between the two groups (Table 3). In addition, patients with genotype TT/TC of rs763110 were found to have remarkably lower expression of FasL than those with genotype CC (0.00022 ± 0.00013 vs. 0.00039 ± 0.00023, p = 0.03). Patients with genotype GG/GA of rs939338 were found to have remarkably higher expression of ABCC5 than those with genotype AA (0.0051 ± 0.0032 vs. 0.0088 ± 0.0049, p = 0.001) (Fig. 2).

Table 3
Association of gene expression with 5-year PFS.

Gene expression	5-year PFS		*p*
	Event (n = 23)	No event (n = 29)	
FasL	0.00025 ± 0.00014	0.00037 ± 0.00021	0.02
ABCC5	0.0084 ± 0.0041	0.0058 ± 0.0027	0.008
MSH2	**0.0042 ± 0.0027**	**0.0053 ± 0.0031**	**0.18**
CASP3	**0.00073 ± 0.00036**	**0.00065 ± 0.00029**	**0.37**
CYP3A4	**0.00085 ± 0.00041**	**0.00093 ± 0.00052**	**0.55**

4. Discussion

Neoadjuvant treatment including cisplatin with doxorubicin, methotrexate, and ifosfamide has been frequently used for osteosarcoma [16–18]. However, differential outcome was observed among patients receiving even the same therapeutic protocol. In previous studies, genetic polymorphisms have been shown to indicate patients' response to chemotherapeutic agents which eventually could influence the final survival [10–13]. Herein, integration of such predictive genetic markers is crucial to optimize risk stratification and personalize chemotherapy strategy. For patients who are predicted to respond poorly to the standardized treatment, alternative or more aggressive therapies could be offered to them for a better outcome.

In this study, we validated a previously reported predictive model that was significantly associated with 5-year PFS of osteosarcoma. Among the 5 variants encompassed in the model, rs763110 and rs939338 were successfully replicated in our cohort of patients. We confirmed that both allele T of rs763110 and allele G of rs939338 were risk factors of decreased 5-year PFS. Based on these two variants, our predictive model can produce a sensitivity of 81.5% and a specificity of 63.3%, respectively. As for the other 3 SNPs, no significant association with the 5-year PFS was found. Specifically, there was no mutant allele of rs4638843 in our cohort. Obviously, the ethnic differences between the Chinese population and the White population could yield great divergence regarding the predictive power of genetic markers. In the study of Hagleitner et al. [14], patients with 5 or more risk alleles can have a 5-year PFS of 41.8%, which was remarkably lower than a 5-year PFS of 100% in patients with no risk allele. Comparably, we observed that patients with allele T of rs763110 and allele G of rs939338 had obviously lower 5-year PFS than those with less than 2 risk alleles. Although having less predictive power as indicated in the current study, the previously reported model was preliminarily validated in our cohort of patients. More causative variants need to be included to increase the reliability of the model.

Identification of the informative gene is critically important for understanding the biological basis of the association between the variants and the clinical outcome. The two informative genes identified in this study were FasL and ABCC5. In previous study, upregulation of the Fas/FasL has been observed in the apoptosis of tumor cells after treatment with chemotherapeutic drugs such as cisplatin [19]. The Fas/FasL system was found almost inactive in cisplatin-resistant cell lines [20]. Moreover, Gordon and Kleinerman [21] reported that FasL

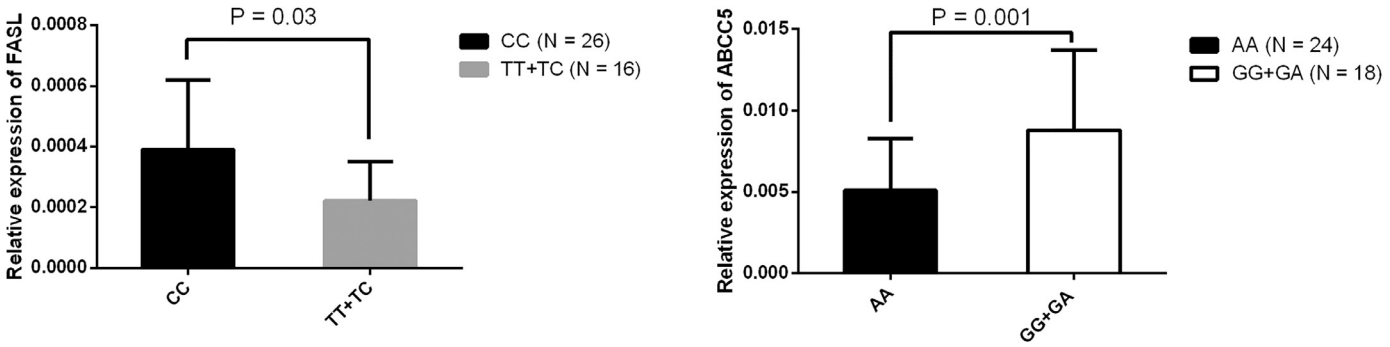

Fig. 2. Relationship between genotypes and gene expression.
For patients with osteosarcoma, genotype TT/TC of rs763110 was indicative of remarkably lower expression of FasL as compared with genotype CC (0.00022 ± 0.00013 vs. 0.00039 ± 0.00023, $p = 0.03$). Genotype GG/GA of rs939338 was indicative of remarkably higher expression of ABCC5 than genotype AA (0.0051 ± 0.0032 vs. 0.0088 ± 0.0049, $p = 0.001$).

expression was inversely correlated with metastatic events of osteosarcoma. In addition to FAS/FASL, previous experiments also showed that higher expression of ABCC5 is associated with the resistance to cisplatin and doxorubicin in different cancer cell lines [22]. In this study, through expression analysis, we found for the first time that the FasL and ABCC5 expression were remarkably correlated with 5-year PFS of osteosarcoma patients. In the luciferase reporter assays reported by Wu et al. [23], allele T of rs763110 was associated with a decreased promoter activity of FASL. The eQTL dataset showed that allele G of rs939338 was linked to a higher expression of ABCC5 [24]. In line with these findings, we confirmed that patients with genotype TT/TC of rs763110 had remarkably lower expression of FasL than those with genotype CC. Moreover, patients with genotype GG/GA of rs939338 were found to have remarkably higher ABCC5 expression than those with genotype AA. Taken together, patients carrying these two risk alleles may benefit less from cisplatin and doxorubicin, which could be taken into account to guide a personalized chemotherapy.

Two limitation of our study should be addressed. As osteosarcoma is a rare disease, the number of patients included in our study is relatively small for a genetic association study. A multi-center study that recruits more patients is warranted to overcome the limitation resulted from the small sample size. The second limitation lies in a paucity of in vitro experiments investigating the influence of the mutant on the cancer cell viability treated by cisplatin or doxorubicin. Future functional studies will provide more clues underlying the relationship between the reported variants and resistance to the chemotherapy drugs.

In summary, the association of FASL and ABCC5 with survival outcome after chemotherapy was validated through a replication study in our cohorts. Moreover, we additionally demonstrated that the expression levels of the identified genes could be indicative of the final outcome. These findings may provide a new insight into a more personalized treatment for patients with osteosarcoma.

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Conflict of interest

No benefits in any form have been or will be received from a commercial party related directly or indirectly to the subject of this manuscript.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jbo.2018.04.003.

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