A first step towards personalized medicine in osteosarcoma: Pharmacogenetics as predictive marker of outcome after chemotherapy based treatment

*Melanie M Hagleitner, Marieke JH Coenen, Hans J Gelderblom, Remco R Makkinje, Hanneke I Vos, Eveline SJM de Bont, Winnette TA van der Graaf, HW Bart Schreuder, Uta Flucke, Peter M Hoogerbrugge, D Maroeska WM te Loo

1Department of Pediatric Hematology and Oncology, Radboud university medical center, Nijmegen, The Netherlands
2Department of Human Genetics, Radboud university medical center, Nijmegen, The Netherlands
3Department of Clinical Oncology, Leiden University Medical Center, The Netherlands
4Department of Pediatric Hematology and Oncology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands
5Department of Medical Oncology, Radboud university medical center, Nijmegen, The Netherlands
6Department of Orthopedic Surgery, Radboud university medical center, Nijmegen, The Netherlands
7Department of Pathology, Radboud university medical center, Nijmegen, The Netherlands
8Department of Clinical Pharmacy & Toxicology, Leiden University Medical Center, The Netherlands

* These authors contributed equally

Corresponding author:
MM Hagleitner,
Department of Pediatric Hematology and Oncology,
Radboud university medical center,
PO Box 9101,
6500 HB Nijmegen,
The Netherlands
T: 0031 24 365 15 45
F: 0031 24 361 93 48
E-mail: Melanie.Hagleitner@radboudumc.nl

Running title: Personalized medicine in osteosarcoma

CONFLICT OF INTEREST: The authors declared no conflict of interest.
Abstract

Purpose: Overall survival in patients with osteosarcoma is only 60%. Poor response to chemotherapy is the dominant risk factor for poor survival. Pharmacogenetic research can offer possibilities to optimize treatment and improve outcome. We applied a pathway-based approach to evaluate the cumulative effect of genes involved in the metabolism of cisplatin and doxorubicin in relationship to clinical outcome.

Experimental Design: We included 126 patients with osteosarcoma. To comprehensively assess common genetic variation in the 54 genes selected, linkage disequilibrium (LD, r²=0.8) based tag-single nucleotide polymorphisms (SNP) strategy was used. A final set of 384 SNPs was typed using Illumina Beadarray platform. SNPs significantly associated with 5-year progression free survival (PFS) were replicated in another 64 osteosarcoma patients.

Results: We identified five variants in Fasl, MSH2, ABCC5, CASP3, and CYP3A4 that were associated with 5-year PFS. Risk stratification based on the combined effects of the risk alleles showed a significant improvement of 5-year PFS. Patients that carried no or only one risk allele had a 5-year PFS of 100% compared to a 5-year PFS of 84.4% for carriers of 2 or 3 risk alleles, 66.7% PFS if a patient carried 4-5 alleles and a 5-years PFS of 41.8% for patients with > 5 risk alleles (p<0.001).

Conclusions: We identified several genes that showed association with PFS in patients with osteosarcoma. These pharmacogenetic risk factors might be useful to predict treatment outcome and to stratify patients immediately after diagnosis and offer the possibility to improve treatment and outcome.

Translational relevance

Osteosarcoma is the most common primary malignant bone tumor in children and young adults. Although outcome improved after the introduction of multi-agent chemotherapy, survival for these patients is still poor. Herein we study whether pharmacogenetic research contributes to a better understanding of the mechanism of drug response in patients with osteosarcoma. We identified genetic variants in genes relevant for the response of chemotherapeutic treatment. With this genetic profile it may be possible to stratify treatment of patients immediately after diagnosis and offer the possibility of a more specific, targeted therapy able to improve survival of patients with osteosarcoma.
Introduction

In the past decades, progression free survival (PFS) rates of patients with osteosarcoma have reached a plateau (1). This is in contrast to other forms of cancer, where clear progress has been made, either by the use of new drugs or the ability of risk stratification and subsequently treatment adjustments (2). Before the introduction of multi-agent chemotherapy in the 1970s, when surgery was the only treatment, approximately 20% of the osteosarcoma patients survived (3). Nowadays, cure rate for non-metastatic osteosarcoma patients approaches 55-65% (4,5). Several factors influence the outcome of patients with osteosarcoma, such as metastatic status, site and size of the tumor, age at diagnosis, surgical approach and response of the tumor to pre-operative chemotherapy (5,6). The absence of active agents for the treatment of osteosarcoma has however, resulted in uniform chemotherapy schedules for all patients. Therefore, new or alternative strategies are urgently needed in order to improve outcome.

Pharmacogenetics might help to stratify patients before start of treatment, as has been shown for patients with other cancer types (7,8). Although a number of studies have been performed into the role of genetic variants associated with the response to treatment in osteosarcoma patients (9-11), none of these studies comprehensively investigated a set of genes involved in the metabolism of cisplatin and doxorubicin, the two main drugs used in treatment of osteosarcoma patients. Hence, we investigated a set of genetic variants in 54 genes known to be involved in the metabolism of these drugs for association with treatment outcome.

Methods

Patients

In the discovery group, 126 patients with osteosarcoma treated in two different centers (Radboud university medical center and University Medical Center Groningen, The Netherlands) were included. The replication cohort consisted of 64 osteosarcoma patients treated at Leiden University Medical Center, The Netherlands. All patients were newly diagnosed, high-grade osteosarcoma with primary localization and/or metastatic disease treated between 1979 and 2008. All patients were of Caucasian origin. Informed consent was according to age obtained from all patients and/or patients’ guardians. The study was approved by the ethics committee of the Radboud university medical center. Two different chemotherapy regimens were used. In the Radboud university medical center, patients diagnosed until 2002 were treated according to an institutional standard therapy consisting of a maximum cumulative cisplatin (600 mg/m²) and doxorubicin (450 mg/m²). After 2002 (21 of 126 patients in discovery group) and all patients of the other two centers (University Medical Center Groningen and Leiden University Medical Center) were treated according to the standard schedule as given in the EURAMOS
study which basically consisted of cisplatin (480 mg/m²)/doxorubicin (450 mg/m²) and additionally high-dose methotrexate (MTX; 96-144 g/m², according to age) (12). Clinical data were collected retrospectively.

In patients alive, DNA was extracted from blood using the QIAamp DNA Blood Midi kit (Qiagen, Venlo, The Netherlands) or saliva (Oragene saliva collection kit; DNA Genotek, Kanata, Ontario, Canada) according to the protocol of the manufacturers. From patients who had died, DNA of paraffin-embedded samples was extracted as recently described (13).

Selection of SNPs and genotyping

Literature (NCBI (PubMed, Gene)) and the Pharmacogenomics Knowledge Base (www.pharmgkb.org) were searched to select representative candidate genes involved in cisplatin and doxorubicin pathway. In the 54 selected genes we investigated SNPs that have been previously associated with functional changes. In addition, selected genes were covered with 5-10 tag SNPs to analyze the complete gene with a minimal set of SNPs. Tag SNP selection was performed in Haploview (LD statistic $r^2 \geq 0.8$, minor allele frequency $> 0.05$) using data from the HapMap project (International HapMap Consortium 2007, HapMap Data Rel24/phaseII Nov08). A final set of 381 SNPs was selected for analysis using the Illumina Beadarray® platform (Supplementary Table 1, Illumina, Inc., San Diego, CA). Illumina Genome Studio software was used for automated genotype clustering and calling. Samples and SNPs with genotyping call rates of < 85% were excluded from analyses, as well as SNPs deviating from Hardy–Weinberg equilibrium ($p$-value $> 0.05$).

Statistical analyses

Statistical differences in demographic data was assessed by the Pearson-chi square test. As this is an exploratory study we considered two-sided $p$-values of $<0.05$ as statistically significant. No multiple testing correction has been applied due to the exploratory nature of the analysis. 5-year Progression free survival (PFS) was defined as the interval between diagnosis and disease progression/recurrence (= event) and was estimated using the Kaplan-Meier method. Patients without disease recurrence at the date of last follow-up were censored at that date. Histologic treatment response was defined as good responders with $< 10\%$ vital cells after pre-operative therapy with two cycles of each drug. Associations between potential confounders (gender, age at diagnosis, metastasis at diagnosis, axial location, MTX as co-medication and histologic response) and PFS or histologic response were evaluated using Cox’s proportional hazard regression analysis or a 2x2 table, respectively. These statistical analyses were performed using SPSS version 20.0 (SPSS Inc., Chicago, III).
To assess the effect of a genetic variant on PFS and histologic response data were dichotomized in event/no event and good/bad responders, respectively. Multivariate logistic regression analyses of PFS and histologic response included MTX exposure (yes/no) in PLINK (14) using the command --logistic (additive model). A meta-analysis of the discovery and replication cohort was performed for the statistically significantly associated variants ($p<0.05$) in the discovery cohort to investigate possible improved association signals. For SNPs with a low heterogeneity fixed effects $p$-values and for SNPs with a high heterogeneity the random effects $p$-value are reported.

To assess the effect of a combination of variants on PFS, a genetic risk score was composed as described in literature (15). The score was constructed based on the number of unfavorable alleles (0-1, 2-3, 4-5 or >5 risk alleles) that were carried by each patient for each of the SNPs associated with PFS. With the risk alleles of each patient we analyzed 5-year PFS curves using the Kaplan-Meier method, also in addition to metastases at diagnose.

**Results**

**Patients**

In total, we included 190 patients with newly diagnosed osteosarcoma. Baseline characteristics did not show statistically significant differences between the discovery and replication groups (Table 1). The number of patients with good histologic response was slightly higher in the replication group ($p=0.07$). This might partially be explained by treatment schedules as patients additionally treated with MTX had a slightly but not significantly better 5-year PFS (66.7% vs. 54.5%, $p=0.13$). Achieving a good histologic response was not influenced by any of the baseline characteristics, such as metastatic state at presentation. No difference in 5-year PFS was seen comparing patients with good histologic response to bad responders (61.3% vs. 55.8%, $p=0.36$). In contrast, 5-year PFS was significantly associated with co-treatment of high-dose MTX. Therefore, MTX exposure was used as covariate in all multivariate analyses. No confounding factor was found for histologic response.

**Association analyses of histologic response**

In total, 177 samples (93.2%) were genotyped successfully, 120 patients (95.2%) in the discovery group and 57 patients (89%) in the replication group (Figure 1: flow chart). The genotype rate in the replication group was smaller due to a higher rate of DNA samples from FFPE material. In the discovery group, 7 SNPs with genotyping call rates < 85%, 31 SNPs deviating from Hardy-Weinberg equilibrium and 111 SNPs with a minor
allele frequency <0.05 in the study population were excluded. In the discovery group, a total of 14 SNPs demonstrated a statistically significant association ($p<0.05$) with histologic response (Supplementary Table 2). Of these, four SNPs (rs11190291, rs723456, rs4645983, rs207455) were excluded because of low genotyping rates in the replication cohort. Meta-analyses of the remaining 10 SNPs, demonstrated 2 statistically significant associations (rs3136326 and rs1800936) however no improved association signal of the discovery and replication cohorts compared to the discovery cohort alone was seen (Supplementary Table 2).

Association analyses of 5-year PFS

Multivariate linear regression analyses corrected for high-dose MTX resulted in the identification of 23 statistically significant ($p<0.05$) SNPs associated with 5-year PFS, in the discovery group. Of these 23 SNPs, nine SNPs (rs3181345, rs4647616, rs1233398, rs2808676, rs3176646, rs2917669, rs1805324, rs1296500, rs1895419) showed low call rates for the replication cohort, leaving 14 SNPs for analysis (Supplementary Table 3). Five SNPs (rs763110, rs4638843, rs939338, rs2720376, rs4646437) demonstrated an improved association signal with PFS in the meta-analysis of the discovery and replication cohorts compared to the discovery cohort (Table 2). These SNPs were not associated with histologic response.

Genetic risk score

Our genetic risk score for PFS was calculated based on the five statistically significantly associated SNPs (rs763110, rs4638843, rs939338, rs2720376, rs4646437) in the meta-analysis of this study, thus, ranging between 0 and 10. Since only a small number of patients had a score of 6 or higher, we merged these groups into one group for analysis, resulting into a total of four groups representing 0-1, 2-3, 4-5 or more than 5 unfavorable alleles.

After adjusting for treatment with MTX, we found that the genetic risk score was associated with 5-year PFS. Patients without or one risk allele showed a PFS of 100% compared to a 5-year PFS of 84.4% with 2-3 risk alleles, 66.7% with 4-5 alleles and 41.8% with > 5 risk alleles ($p<0.001$, Figure 2). Adding the genetic risk score to the presence of metastases at diagnosis, the most important conventional risk factor, significantly improved the ability to predict PFS. The 5-year PFS of patients without metastases at diagnosis showed similar PFS as the complete population of 100% ($p=0.008$). In patients with metastases at diagnosis, all patients were carriers of at least 2 risk alleles. The 5-year PFS of patients with 2-3 risk alleles and metastases at diagnosis was still 80%. However, PFS decreased to 56.2% in patients carrying 4-5 risk alleles and only 12.5% in patients with > 5 risk alleles ($p=0.007$; Figure 3).
Discussion

In this study we showed that the pharmacogenetic profiles of patients with osteosarcoma may be used to distinguish patients with good outcome from patients with poor outcome. Based on our analysis five genetic variants were significantly associated with 5-year PFS. To our knowledge this is the first pharmacogenetic study in osteosarcoma patients that demonstrate that it is possible to perform risk stratification at start of treatment for this specific patient group.

Interestingly, good histological response which is a highly prognostic factor for survival in most studies was not associated with an increased 5-year PFS in this study. We are not the first showing limited value of histological response for treatment stratification. Several trails have shown that intensification of chemotherapy preoperative resulted in a significant higher proportion of good responders, but outcome was similar (16-18). Nevertheless, we investigated whether germline genetic polymorphisms can predict histologic response to pre-operative treatment. None of the identified SNPs in the discovery group showed an improved signal in the meta-analysis of both cohorts. Two of the associated SNPs remained significant though with higher p-values compared to the discovery cohort. These SNPs might represent true associations as our replication cohort is relatively small, we advise that our findings should be validated in a larger cohort.

We identified one functional SNP in the Fas Ligand (*FasL*) gene and four tag-SNPs in the *MutS homologue 2* (*MSH2*); *Caspase 3* (*CASP3*); of the *ATP-binding cassette, sub-familiy C, member 5* (*ABCC5*) and *Cytochrome P450 3A4* (*CYP3A4*) to be associated with a lower PFS ([Supplementary Table 3](#)).

*FasL* induces apoptosis and appears to have a role in the development and progression of cancer (19). The associated SNP, rs763110 (844T>C) in *FasL*, is suggested to influence FASL expression (20). Fas expression correlates inversely with metastatic potential in human osteosarcoma cells (21). This is in accordance with our findings where the T allele, linked to a lower FasL expression, is associated with unfavorable disease outcome. It is known that chemotherapy upregulates Fas expression (22), therefore, patients carrying the T allele might benefit from co-treatment with an agent that additionally up-regulates FasL expression.

The *MSH2* gene encodes for the MSH2 protein which is part of the MutSα complex in the Mismatch Repair (MMR) system. This system recognizes cisplatin-induced DNA damage. A recent study on osteosarcoma tumor tissue showed that MSH2 expression was significantly associated with non-response to chemotherapy and a decreased overall survival (23). The functional effect of the identified variant is not known, however, it is thinkable that this variant has an effect on the DNA repair capacity leading to a decreased survival.
We also identified a variant in *ABCC5* associated with an unfavorable 5-year PFS. It has been demonstrated that elevated expression of *ABCC5* (or MRP5) confers resistance to cisplatin and doxorubicin in different cell lines (24,25). A search for eQTL effects of the SNP showed indeed that the variant regulates the expression of *ABCC5*, with the A allele linked to a lower expression (26). In this study the G allele of rs939338 showed association with a decreased PFS which indicates that carriers of this variant might have a higher expression of *ABCC5* leading to more resistance to chemotherapy.

Another association found represents a variant in *CASP3* which is one of the main executor genes in cell apoptosis. A search in a blood eQTL dataset showed that the C allele of this variant is linked with lower CASP3 gene expression (26). Association studies including *CASP3* are scarce but, as *CASP3* is at the heart of the apoptotic process it is reasonable to hypothesize that genetic variation in this gene can influence on treatment outcome.

The last association identified was a variant in *CYP3A4* which is involved in oxidation of cisplatin but also plays a role in the doxorubicin pathway. CYP3A enzymes inactivate many anticancer drugs, overexpression of CYP3A in tumors could result in an increased drug inactivation and decreased drug efficacy. In osteosarcoma tumors, indeed expression of CYP3A4 was found to be significantly higher in the tumor of patients who developed distant metastatic disease compared to patients with progression free survival (27,28). Furthermore, cell lines overexpressing CYP3A4 exhibit an increased resistance to doxorubicin (29). In our study, the associated variant had a positive effect on PFS suggesting that this variant might result in a low CYP3A4 expression and therefore, less resistance to therapy.

This pharmacogenetic analysis is the first in literature to report on a combined effect of genetic variants using a genotype risk score in osteosarcoma patients. To evaluate our findings we added conventional risk factors, such as metastases at diagnosis to the SNP panel. Interestingly, in both patients with or without metastases at diagnosis we were able to distinguish patients with good outcome from patients with poor outcome based on the genetic risk score. Interestingly, all patients with metastasis carried at least more than two unfavorable alleles, suggesting that the genes which were initially selected to be related with the metabolism of doxorubicin and cisplatin might also influence the development of metastasis. For all associated genes in this study we have identified publications suggesting that the expression of these five genes are linked to metastasis formation (20,22,27,30,31). We were able to identify a group of patients with very poor outcome which may benefit from alternative or novel treatment strategies. However, we realize our findings bring new questions. The included genes were chosen for their relationship to the metabolism of doxorubicin and cisplatin. Both drugs have dose
limiting toxicities. So what to do with patients with predicted poor outcome? Can we risk to give higher doses of chemotherapy and if not, what could be an alternative strategy?

Although the results of our study are promising, we must acknowledge that our cohort was heterogeneous with the inclusion of patients with both, metastatic and localized disease. Furthermore, some patients received a two drug and some a three drug regimen which might have led to an increased histologic response rate to the pre-operative chemotherapy dose, but was not translated into better survival. In addition, the study should be considered with caution as we did not correct for multiple testing and the number of patients included is limited. However, osteosarcoma is a rare disease and, at present, no other pharmacogenetic multidrug candidate pathway gene study in literature is available that includes a cohort as large as ours (n=177). Post-hoc power analysis shows that we had more than 80% power to detect associations for the 5-year PFS analysis with an odds ratio of 1.5 for heterozygotes (minor allele frequency >0.35, p-value 0.0036 (correction for 14 SNPs)) (32). Including this study, a total of four outcome-related pharmacogenetic studies in patients with osteosarcoma have been published (9-11). However, different treatment protocols resulted in different strategies to select genes for analyses. As a consequence, most of the previously reported genes were not included in this study.

In conclusion, this exploratory study identifies several genes that might be associated with treatment response in patients with osteosarcoma. Replication of our data in additional cohorts and prospective clinical studies in a more homogenous group is warranted to reaffirm the potential of using pharmacogenetics in patients with osteosarcoma and bring about a more personalized treatment for these patients.

References


Tables and Figures:

Table 1. Demographic data

Table 2. Genetic variants associated with 5-year PFS

Figure 1. Flow chart

Figure 2. 5-year PFS based on genetic risk score

Figure 3. 5-year PFS based on genetic risk score, analysis split based on the presence of metastases at diagnosis
Table 1. Demographic data

<table>
<thead>
<tr>
<th></th>
<th>Total (N=190)</th>
<th>Discovery (N=126)</th>
<th>Replication (N=64)</th>
<th>p-value&lt;sup&gt;1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (years; median)</td>
<td>16.9 (2.8-67.3)</td>
<td>15.6 (2.8-39.5)</td>
<td>18.2 (5.0-67.3)</td>
<td>0.5</td>
</tr>
<tr>
<td>Male gender (N; %)</td>
<td>99 (52.1%)</td>
<td>67 (53.2%)</td>
<td>32 (50%)</td>
<td>0.68</td>
</tr>
<tr>
<td>Primary metastases (N; %)</td>
<td>39 (20.5%)</td>
<td>26 (20.6%)</td>
<td>13 (20.3%)</td>
<td>0.96</td>
</tr>
<tr>
<td>Tumor in axial skeleton (N; %)</td>
<td>13 (6.8%)</td>
<td>9 (7.1%)</td>
<td>4 (6.2%)</td>
<td>0.36</td>
</tr>
<tr>
<td>Poor histologic response (N; %)</td>
<td>112 (66.3%)</td>
<td>77 (71.3%)</td>
<td>35 (57.4%)</td>
<td>0.07</td>
</tr>
<tr>
<td>5-year PFS&lt;sup&gt;2&lt;/sup&gt;</td>
<td>60.5%</td>
<td>57.9%</td>
<td>65.6%</td>
<td>0.43</td>
</tr>
</tbody>
</table>

<sup>1</sup>comparing discovery vs. replication group

<sup>2</sup>patients available for analyze: Total cohort: N=115. Discovery: N=73. Replication: N=42.
Table 2. Genetic variants associated with 5-year PFS

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>Allele</th>
<th>P</th>
<th>OR</th>
<th>95% CI</th>
<th>Meta analyse fixed effects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Discovery group</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P</td>
</tr>
<tr>
<td>FasL</td>
<td>rs763110</td>
<td>T</td>
<td>0.04</td>
<td>1.97</td>
<td>1.04-3.73</td>
<td>0.02</td>
</tr>
<tr>
<td>MSH2</td>
<td>rs4638843</td>
<td>C</td>
<td>0.04</td>
<td>2.32</td>
<td>1.02-5.27</td>
<td>0.007</td>
</tr>
<tr>
<td>ABCC5</td>
<td>rs939338</td>
<td>G</td>
<td>0.03</td>
<td>1.86</td>
<td>1.06-3.24</td>
<td>0.03</td>
</tr>
<tr>
<td>CASP3</td>
<td>rs2720376</td>
<td>G</td>
<td>0.02</td>
<td>0.52</td>
<td>0.30-0.90</td>
<td>0.01</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>rs4646437</td>
<td>A</td>
<td>0.02</td>
<td>0.34</td>
<td>0.13-0.85</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Multivariate linear regression analyses corrected for high-dose MTX.
Figure 1.

**Discovery**

- 126 patients with osteosarcoma:
  - 6 samples excluded with genotype rate < 80%
- 381 SNPs genotyped:
  - 7 SNPs excluded due to low genotype rate (<85%)
  - 31 SNPs failed to meet Hardy-Weinberg equilibrium (HWE)
  - 111 SNPs excluded with allele frequency < 0.05
- 232 SNPs in 120 patients remained for further analyses

SNPs with p < 0.05
- Histologic response: 14 SNPs
- 5-year PFS: 23 SNPs

**Replication**

- 64 patients with osteosarcoma:
  - 7 samples excluded with <80% genotype rate
- 14 SNPs with improved p-value for histologic response
  - 4 SNPs excluded due to low genotype rate (<85%)
- 23 SNPs with improved p-value for 5-year DFS
  - 9 SNPs excluded due to low genotype rate (<85%)

Meta analyses:
- Histologic response: No SNP associated
- 5-year PFS: 5 SNPs
Patients without or one risk allele showed a PFS of 100% compared to a 5-year PFS of 84.4% with 2-3 risk alleles, 66.7% with 4-5 alleles and 41.8% with > 5 risk alleles (p<0.001)
Patients carrying no or one risk allele had a PFS of 100% compared to a 5-year PFS of 85% in patients with 2-3 risk alleles, 69.8% with 4-5 risk alleles and 53.8% in patients with > 5 risk alleles (p=0.008).

Patients with metastases and 2-3 risk alleles had a PFS of 80% compared to 56.2% in patients carrying 4-5 risk alleles and 12.5% in patients with > 5 risk alleles (p=0.007).
A first step towards personalized medicine in osteosarcoma: Pharmacogenetics as predictive marker of outcome after chemotherapy based treatment

Melanie M Hagleitner, Marieke JH Coenen, Hans Gelderblom, et al.

*Clin Cancer Res* Published OnlineFirst March 31, 2015.