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Pharmacogenetics of bisphosphonate-associated osteonecrosis of the jaw

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1. ABSTRACT

An undesirable effect associated with bisphosphonates is osteonecrosis of the jaw (ONJ). Case reports discussed ONJ development in patients with multiple myeloma or metastatic cancers receiving bisphosphonates as palliation for malignant bone disease. No causative relationship has been unequivocally demonstrated between ONJ and bisphosphonate therapy. To determine if a higher sensitivity to bisphosphonates could in part explain the development of ONJ, the segregation of *A/C rs2297480* polymorphism of gene encoding for the farnesyl pyrophosphate synthase (FDPS) with ONJ was evaluated in a cohort of 68 Caucasian patients treated with zoledronic acid for multiple myeloma and metastatic mammary and prostate cancer. The AA and CC genotypes were highly differently distributed among ONJ patients and controls, matched for sex and type of malignant disease, with a positive correlation between AA carrier status and occurrence of ONJ ($p=0.03$) after 18-24 months of treatment. Because *FDPS* gene variants have been associated with bone morbidity, these pharmacogenetic association likely reflect the interaction of amino-bisphosphonates with germline sensitivity to drug actions, and might identify patients at highest risk to develop ONJ.

2. INTRODUCTION

Osteonecrosis of the jaw (ONJ) has been recognized as an uncommon and severe event with exposed bone in the mandible, maxilla or both that persists for at least eight weeks, in the absence of previous radiation and of metastases in the jaws (1,2). During the past five years, a number of letters, case reports, and case series published in the medical literature have discussed the association of ONJ with the use of bisphosphonates. The majority of cases have occurred in patients treated for 1.5 to 3 years with high doses of nitrogen-containing intravenous bisphosphonates as adjunctive therapy for multiple myeloma or metastatic cancer, usually after a dental surgical procedure on areas more prone to intraoral trauma (3). The efficacy of bisphosphonates in reducing bone pain, hypercalcaemia, and skeletal complications has been extensively documented in patients with advanced metastatic cancer (4). If tolerated, it is not uncommon for these patients to be maintained on bisphosphonate therapy indefinitely, although, duration of therapy appears to be the most significant risk factor for ONJ development (5).

As the risk of ONJ in patients treated with high doses of intravenous bisphosphonates is high (in the range of 1-10 per 100 patients) and because the management of

this condition is largely supportive, Regulatory Agencies now require a precaution regarding ONJ risk in package inserts for all nitrogen-containing bisphosphonates. Patients who are to receive intravenous bisphosphonates therapy for cancer should also see their dentists, following a regular program of oral and dental maintenance (6,7).

Even though a clear pathogenetic mechanism by which bisphosphonates cause ONJ is not established, it is hypothesized that suppressed bone turnover caused by amino-bisphosphonates leads to decreased bone flow, to cell necrosis and apoptosis and to damage of oral epithelium (8-10). Other predisposing factors appear to be dental diseases, dental surgery, oral trauma, periodontitis, and poor dental hygiene (6,7). Therapy with cytostatic agents and corticosteroids, common in cancer patients, can contribute to increase the risk of infection. These conditions, together with the effects of bisphosphonates on T-cell function, can predispose patients to become immunocompromised, with consequent delayed healing of the oral lesions. Pathogenesis of ONJ may also involve other factors, including systemic diseases (i.e. diabetes) and the lack of use of calcium and vitamin D therapy in patients treated with high doses of amino-bisphosphonates for cancer (11). Since the doses used in the adjuvant therapy of multiple myeloma and of metastatic cancer are standard, but not all the patients develop this disorder, the phenotypic characteristic of the single patients can play an important role in favouring the development of this severe adverse event.

Another approach to the understanding of the etiology of adverse events due to pharmacological therapies is the genetic diversity among treated patients. Indeed, pharmacogenetics could be helpful in the early recognition of individuals at risk to develop adverse drug reactions, such as acute phase symptoms, ONJ, and atrial fibrillation (12). Pharmacogenetic analysis of genes encoding the enzymes of the mevalonate pathway (i.e. the amino-bisphosphonate natural targets) could represent a useful approach to answer these critical questions. In a recent study carried out by our group in a cohort of Caucasian women enrolled in osteoporosis controlled clinical trials with amino-bisphosphonates, we demonstrated that a polymorphism of the farnesyl pyrophosphate synthase (*FDPS*) gene segregates with a different response of bone turnover markers to the antiresorptive therapy (13).

In the present study we performed an exploratory analysis to assess whether a common polymorphism in the target *FDPS* gene is associated with the risk to develop ONJ in a cohort of Caucasian patients treated with zoledronic acid for multiple myeloma, for metastatic mammary and for prostate cancer.

3. MATERIALS AND METHODS

3.1. Patients

This was a retrospective analysis of 68 between myeloma and metastatic cancer patients (16 myeloma females, 20 myeloma males, 22 metastatic mammary cancer females and 10 metastatic prostate cancer males) evaluated at the Metabolic Bone Diseases Unit and at the Dental School of the University of Florence, Italy. The characteristics of the ONJ patients and of the controls are reported respectively in Tables 1 and 2. ONJ patients (18 females and 16 males) and control patients (20 females and 14 males), matched for sex and type of cancer, were included in the genetic study if their dental and medical data could be retrieved and verified and if they had a dental assessment, including oral examination with panoramic radiographs. Patients who had a prior history of radiation therapy to the jaw region or neoplastic disease that directly involved the jaws were excluded from the genetic analysis. All the patients had been treated with zoledronic acid (Zometa R) administered intravenously as a monthly infusion at a dose of 4 mg for a period ranging from 18 to 24 months, during the same period (July 2004 to December 2007), irrespective to the date of diagnosis of the malignant disease. All patients were actively receiving chemotherapy. The individual chemotherapeutic regimens varied widely in accordance with tumor type and characteristics. The Institutional Review Board at the University Hospital of Florence approved the study and all the patients received an informed consent.

The following data were collected for each patient: demographics, chemotherapeutic treatment, the presence of lytic bone disease by radiologic assessment, length of treatment with intravenous bisphosphonates, and dental intervention for ONJ. Panoramic radiographs were assessed in every patient, including the control group.

3.2. Genetic analysis

Genomic DNA was extracted from peripheral blood leukocytes using NucleoSpin Blood Quick Pure microvolume extraction method (Macherey-Nagel, Easton, PA, USA) according to the manufacturer's instructions. The DNA region containing the *A/C rs2297480* polymorphism of the *FDPS* gene was amplified by polymerase chain reaction (PCR) in a final volume of 50 µl containing 1X of reaction buffer, 0.4. µM of each primer, 0.2. mM of dNTPs, 1 U of GoTaq DNA Polymerase (Promega, Madison, WI, USA) and about 50 ng of genomic DNA. Thermal cycling conditions were 94^{o} C for 3 min, 35 cycles of 94^{o} C for 30 sec, 54^{o} C for 30 sec and 72^{o} C for 30 sec, followed by an additional 72^{o} C for 5 min stabilization step. Each PCR product (324 bp) was digested at 37° C for 5 hours with 1 U of *SmuI* (*FauI*) endonuclease (MBI Fermentas, Vilnius, Lithuania). Digestion products were visualized by 3% ethidium bromide stained agarose gel electrophoresis. Fragments were separated depending on their length revealing presence (C allele) or absence (allele A) of the restriction site.

3.3. Statistics

Patients' characteristics (age and months of treatment) have been evaluated in the ONJ and in the control groups and expressed as mean plus/minus standard deviation (SD). *T* Student test was used to estimate differences in the mean age and in the mean treatment exposure between the ONJ and control groups. Differences were considered significant in the p-value was less than 0.05.

Table 1. ONJ patients' characteristics

Patient No	Gender	Age (years)	Diagnosis	Treatment Exposure (months)	Site of Necrosis	Surgical Intervention	*FDPS* genotype
1	F	68	Myeloma	18	Maxilla	Partial maxillectomy	AA
2	F	63	Myeloma	19	Mandible	Sequestrectomy	AC
3	F	57	Myeloma	18	Mandible	Sequential mandibulectomy	AA
4	F	76	Myeloma	20	Maxilla	Conservative treatment	AA
5	F	79	Myeloma	22	Bilateral maxilla	Multiple sequestrectomy	AC
6	F	58	Myeloma	24	Bilateral mandible	Sequential mandibulectomy	AA
7	F	59	Myeloma	18	Mandible (pathological fracture)	Sequential mandibulectomy	AC
8	F	63	Myeloma	19	Bilateral mandible	Sequestrectomy	AC
9	F	47	Breast cancer	21	Maxilla	Sequestrectomy	AA
10	F	77	Breast cancer	20	Bilateral mandible	Sequestrectomy	CC
11	F	55	Breast cancer	18	Mandible	Sequestrectomy	AC
12	F	72	Breast cancer	18	Mandible	Sequestrectomy	AA
13	F	54	Breast cancer	18	Bilateral maxilla	Sequestrectomy	AA
14	F	63	Breast cancer	22	Maxilla	Partial maxillectomy	AC
15	F	58	Breast cancer	23	Maxilla	Sequestrectomy	AA
16	F	72	Breast cancer	24	Maxilla	Partial maxillectomy	AC
17	F	70	Breast cancer	18	Mandible	Sequestrectomy	AA
18	F	69	Breast cancer	19	Maxilla	Sequestrectomy	CC
19	M	72	Myeloma	19	Bilateral maxilla	Sequestrectomy	AA
20	M	76	Myeloma	20	Maxilla	Sequestrectomy	AC
21	M	70	Myeloma	22	Mandible	Sequestrectomy	AA
22	M	68	Myeloma	24	Mandible	Sequestrectomy	AA
23	M	57	Myeloma	23	Bilateral maxilla	Sequestrectomy	AC
24	M	64	Myeloma	20	Mandible	Sequential mandibulectomy	AA
25	M	76	Myeloma	18	Mandible	Sequestrectomy	AC
26	M	63	Myeloma	19	Maxilla	Sequestrectomy	AA
27	M	70	Myeloma	20	Bilateral maxilla	Sequestrectomy	AA
28	M	58	Myeloma	22	Mandible (pathological fracture)	Sequential mandibulectomy	AA
29	M	67	Prostate cancer	21	Maxilla	Sequestrectomy	AC
30	M	71	Prostate cancer	18	Maxilla	Conservative treatment	AA
31	M	65	Prostate cancer	20	Mandible	Sequestrectomy	AA
32	M	63	Prostate cancer	18	Mandible	Sequential mandibulectomy	AA
33	M	70	Prostate cancer	18	Maxilla	Conservative treatment	AA
34	M	65	Prostate cancer	21	Mandible	Sequestrectomy	AA

Table 2. Control patients' characteristics

Patient n.	Gender	Age (years)	Diagnosis	Treatment Exposure (months)	*FDPS* genotype
35	F	65	Myeloma	20	CC
36	F	58	Myeloma	18	AC
37	F	72	Myeloma	23	AC
38	F	70	Myeloma	18	AA
39	F	71	Myeloma	20	CC
40	F	48	Myeloma	19	CC
41	F	55	Myeloma	22	AC
42	F	58	Myeloma	24	AA
43	F	67	Breast Cancer	21	AA
44	F	63	Breast Cancer	20	AC
45	F	64	Breast Cancer	18	AC
46	F	75	Breast Cancer	22	AA
47	F	71	Breast Cancer	18	AC
48	F	64	Breast Cancer	19	CC
49	F	69	Breast Cancer	23	AC
50	F	74	Breast Cancer	18	AC
51	F	60	Breast Cancer	20	AA
52	F	61	Breast Cancer	21	AA
53	F	70	Breast Cancer	19	AC
54	F	65	Breast Cancer	18	AA
55	M	70	Myeloma	22	AC
56	M	65	Myeloma	20	AA
57	M	61	Myeloma	18	AA
58	M	75	Myeloma	19	CC
59	M	78	Myeloma	20	AC
60	M	64	Myeloma	23	AA
61	M	69	Myeloma	21	AA
62	M	68	Myeloma	18	CC
63	M	73	Myeloma	18	AC
64	M	77	Myeloma	22	AC
65	M	60	Prostate Cancer	20	AC
66	M	58	Prostate Cancer	21	AA
67	M	65	Prostate Cancer	19	CC
68	M	71	Prostate Cancer	20	CC

Table 3. ONJ patients' characteristics expressed as mean plus/minus standard deviation (SD).

Women mean age (years)	Men mean age (years)	General mean age (years)	Women treatment Exposure (months)	Men treatment Exposure (months)	General treatment Exposure (months)
64.44 ±8.94	67.19 ± 5.54	65.73 ± 7.55	19.94 ± 2.18	20.19 ± 1.87	20.06 ± 2.01

Table 4. Control patients' characteristics expressed as mean plus/minus standard deviation (SD).

Women mean Age (years)	Men mean Age (years)	General mean Age (years)	Women treatment exposure (months)	Men treatment exposure (months)	General treatment exposure (months)
65.00 ± 6.90	68.14 ± 6.30	66.29 ± 6.75	20.05 ± 1.93	20.07 ± 1.70	20.06 ± 1.77

Table 5. Results of *t* Student test application to mean age and mean treatment exposure differences among ONJ and control groups

Mean age (years)	Mean treatment exposure (months)
Women	
$t = 0.2156$ p = 0.83	$t = 0.1582$ p = 0.87
Men	
$t = 0.4421$ p = 0.66	Men $t = 0.1816$ p = 0.86
General	
$t = 0.4850$ p = 0.63	$t = 0.1962$ p = 0.84

Table 6. Hardy-Weinberg equilibrium of *FDPS* genotype distribution in analyzed sample population with respect to Caucasian population

Allele frequencies in Caucasian population	Observed genotype distribution	Expected genotype distribution	
A allele = 0.66	AA = 33	AA = 29.62	
C allele = 0.34	AC = 25	AC = 30.74	$\chi^2 = 1.923$
	CC = 10	CC = 7.86	

Table 7. Relationship between *FDPS* genotypes and bisphosphonate-related ONJ occurrence

FDPS genotypes	ONJ cases n. (%)	Control cases n. (%)	*p*
AA	21 (61.8)	12 (35.3)	**0.033**
Others	13 (38.2)	22 (64.7)	
CC	2 (5.9)	8 (23.5)	**0.045**
Others	32 (94.1)	22 (76.5)	

Statistical analysis to evaluate correlation between genotypes of the *FDPS* gene polymorphisms and occurrence of ONJ was determined using the Fisher Exact Test via the SPSS Inc. software (version 13). Statistical significance was defined as p-value <0.05.

4. RESULTS

4.1. Patient demographics

The general characteristics of the patients who developed ONJ and of those who did not developed this complication are depicted respectively in Tables 1 and 2. The mean age and the mean treatment exposure to zoledronic acid treatment for ONJ patients and for controls are depicted respectively in Tables 3 and 4. No significant differences were found in mean age and mean treatment exposure between the control and ONJ groups (Table 5).

4.2. Genotype/phenotype correlations

Analyzed population, even if small-sized, resulted to be in Hardy-Weinberg equilibrium with respect to the Caucasian population (13,14), (Table 6). Genotype frequencies of the *A/C rs2297480* polymorphism in the ONJ group were distributed as it follows: AA = 21 (61.8 %), AC = 11 (32.3%) and CC = 2 (5.9%), showing that the A allele frequency is 0.78, while the C allele frequency is 0.22, with a heterozygosity index of 0.32. Conversely, in the control group the genotype frequencies were: AA = 12 (35.3 %), AC = 14 (41.2%) and CC = 8 (23.5%), showing that the A allele frequency is 0.5.6, while the C allele frequency is 0.44, with a heterozygosity index of 0.41. The occurrence of a relationship between *FDPS* genotypes and ONJ was statistically evaluated, with the demonstration of a different distribution of *FDPS* AA and CC genotypes between ONJ cases and controls matched for sex, oncological disease and length of treatment. AA carriers were about 62% in ONJ cases and 35% in controls (Table 7). Conversely, *FDPS* gene CC carriers were approximately 6% in ONJ patients and 24% in controls (Table 7). Consequently, *FDPS* AA and CC genotypes' distribution was correlated with bisphosphonate-related ONJ occurrence versus any other genotype and there was a positive correlation between AA carrier status and disease expression (p=0.033), as well as between CC carrier status and the absence of the ONJ complication after 18-24 months of intravenous zoledronic acid treatment (p=0.045).

5. DISCUSSION

It has been well established that intravenously administered bisphosphonates are extremely effective in reducing the symptoms and complications of metastatic bone disease, with a profound impact on the quality of life of these patients. Thus bisphosphonates are frequently prescribed as adjuvant therapy to patients with osteolytic metastases, especially if there is risk for significant morbidity, resulting in a rampant use of these compounds in most medical oncology practices within the past several years. However, the jaw complications recognized as ONJ had a major negative impact on the quality of daily life in these patients. Even though the etiology of this complication is unclear, it appears that segregation with

bisphosphonate treatment exists (5). Since bisphosphonate-associated ONJ is a relatively recently described clinical entity, it is recommended that a consistent case definition, a minimal reporting requirement and a hierarchy of evidence is to be used for subsequent reporting of the disorder. Moreover, more information is needed on the major risk factors for developing this complication. The research agenda is certainly busy.

Patients treated for metastatic bone diseases are a heterogeneous population with uncertainties in treatment regimes and with variable co-morbidities that may influence the response to therapy. Risk factors for ONJ have not been studied in detail and remain speculative, but are likely to include trauma to the oral cavity, use of immunosuppressive drugs, co-morbid diseases such as rheumatoid arthritis and diabetes, cumulative exposure to bisphosphonates, secondary hyperparathyroidism, and poor oral hygiene. Another difference with potential influence on the response to therapy is the genotype of a given patient.

In a recent report bisphosphonate-related ONJ was shown to be associated with polymorphisms of the cytochrome P450 CYP2C8 in multiple myeloma through a genome-wide single nucleotide polymorphism analysis (15). However, because bisphosphonates do not undergo any physical-chemical modifications, variability in gene encoding CYP2C8 would not play a role in their metabolism. Therefore, there is no direct relationship between CYP2C8 and the pharmacogenetic profile of amino-bisphosphonates, even though in a recent report alendronate was shown to mediate suppressive effects on CYP2B (16). Achieving on understanding of the underlying mechanisms will enhance our knowledge about the biological capacity of amino-bisphosphonates to influence the cytochrome molecules.

A good opportunity to analyze this theoretical proposal may derive from the evaluation of genes' variants segregating with the bisphosphonate-induced expression of the ONJ complication. Bearing these considerations in mind, we have explored the *A/C rs2297480* polymorphism of the *FDPS* gene, the gene encoding for the farnesyl pyrophosphate synthase enzyme, in a population of patients affected by metastatic bone disease and treated with zoledronic acid for 18-24 months. The results obtained were compared with *FDPS* gene variant distribution in a control population of cancer patients (matched for tumor type, length of zoledronic acid treatment and sex distribution), who did not develop symptoms and signs of ONJ.

Interestingly, the intronic *A/C rs2297480 FDPS* gene polymorphism analyzed in the present study was recently shown to segregate with a different response of bone turnover markers to amino-bisphosphonate therapy in a Caucasian female population (13). In this study the most responsive genotype (AA) showed a frequency of about 57.7 %, with the opposite CC genotype being the least represented (5.6%). As a potential risk factor for ONJ is over-suppression of bone turnover, the possibility that a genotype more sensitive to the action of bisphosphonates could play a role in the development of the ONJ complication would be extremely fascinating.

Overall, results from the present study demonstrated differences in the allele and genotype frequencies of the *A/C rs2297480 FDPS* gene polymorphism between ONJ cases and controls. These results are in agreement with the previously described higher responsivity of the AC and AA genotypes to oral treatment with amino-bisphosphonates when compared to the CC genotype (13). It could be assumed that the A allele segregates with the ONJ complication through a positive modulation of the response to a potent amino-bisphosphonate, as zoledronic acid.

The functional consequences of the intronic A/C *rs2297480 FDPS* gene polymorphism have not been fully elucidated, but recent *in silico* analysis of this polymorphism revealed that the A allele may create a binding site for Runx1, a transcription factor expressed in mouse osteoclasts and osteoclast precursors (17). It could be hypothesized that Runx1 may bind to the *FDPS* promoter site, created by the A allele, and thus reduce osteoclast activity by inhibiting *FDPS* transcription. However, further *in vitro* studies are necessary to show the level of *FDPS* transcription inhibition by the A allele.

The person-to-person variability of a drug response represents a major problem in clinical practice and in drug development, as the great majority of commonly used drugs are effective in a variable percentage of all treated patients. Moreover, some patients develop adverse drug reactions that represent the fourth case of hospitalization and the sixth cause of death in the US. Through pharmacogenetics, the influence of genetic variation on individual drug response is explained by correlating DNA polymorphisms and/or gene expression with drug's efficacy or toxicity. All the pharmacogenetic applications require the identification and validation of a genetic marker to be investigated in patients non-responding to a therapy or at risk of adverse reactions (18). At this stage the A/C *rs2297480 FDPS* gene polymorphism can be classified as "probable valid", because it appears to have predicted value but it has not been yet replicated or widely accepted.

The steps for the validation of *FDPS* gene polymorphism as a genetic biomarker to predict the development of the ONJ complication in patients treated with zoledronic acid will encompass: 1) studies aimed to increase sensitivity, reliability and specificity of the assay; 2) the evaluation of the variability in human populations to determine relevant interactions and potential confounders; and 3) studies that aim to establish the possibility of a causal relationship between this biomarker candidate and the response to zoledronic acid and to other amino-bisphosphonates.

Although conducted in a small-sized sample, this work represents the first study on the segregation of ONJ development with genomic variants. As there is a

difference between the distribution of *FDPS* gene polymorphisms in Caucasian and Asian populations, with the *rs2297480* CC genotype being the least represented in Caucasians and the most represented in Asians (13,19), future studies should encompass different ethnic groups. Moreover, the ONJ pharmacogenetic studies will include other polymorphisms within the *FDPS* gene and/or other genes of the mevalonate pathway. With only 68 patients and one polymorphism analyzed, there is a risk for type I error, that is the relationship we observed were due to chance, or type II error, that we missed some important pharmacogenetics determinants. Moreover, as opposed to a genome-wide approach, we have interrogated a locus that has been associated with clinical functional consequences (13).

In conclusion, this is the first report that analyses a gene in the mevalonate pathway as a pharmacogenetic biomarkers to be added to the set of risk factors that underlie ONJ pathogenesis. No direct causative relationship has been demonstrated between ONJ and *FDPS* gene polymorphisms. However, the *rs2297480* A/C pharmacogenetic polymorphism associated with ONJ has biological plausibility, and if confirmed in other patient cohorts, could provide the foundation for future dosage individualization, based on simple genetic tests. Since the incidence of ONJ in patients treated with intravenously administered aminobisphosphonates is elevated, prospective analyses in large populations and in different ethnic group are recommended in this condition.

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Abbrevations: ONJ: osteonecrosis of the jaw, FDPS: farnesyl pyrophosphate synthase, SD: standard deviation

Key Words: Bisphosphonates; Osteonecrosis; Metastases; Mevalonate Pathway; Gene Polymorphisms; Pharmacogenetics, Review

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