

RESEARCH ARTICLE

Decreased HDL-Dependent Paraoxonase and Arylesterase Enzyme Activity May Indicate a Worse Prognosis in Multiple Myeloma

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Abstract

Background: Multiple myeloma (MM) is a haematological cancer characterized by clonal proliferation of plasma cells. The aim of this study was to investigate the activity of serum paraoxonase-1 (PON1) and arylesterase (ARE) in multiple myeloma with and without free light chain excretion (FLCe-MM and NFLCe-MM); as well as to investigate possible alterations in oxidative stress parameters. **Materials and Methods:** Total thiol (T.thl), oxidative stress index (OSI), total oxidant status (TOS) and total antioxidant status (TAS) were examined in addition to the PON1 and ARE enzyme activities in twenty one FLCe-MM and nineteen NFLCe-MM subjects. Routine parameters like lipid panel, serum total protein, albumin, creatinine, blood urea nitrogen (BUN), uric acid and hemoglobin levels were compared with the oxidative stress markers. **Results:** Serum total protein, BUN, creatinin, and uric acid levels were significantly higher ($p=0.04$, $p=0.001$, $p=0.001$ and $p=0.0022$, respectively), while hemoglobin and albumin levels were significantly lower in FLCe-MM patients ($p=0.009$ and $p=0.04$, respectively). PON1 and ARE activities were significantly lower in patients with FLCe-MM compared to those with NFLCe-MM ($p=0.001$ and $p=0.008$, respectively). **Conclusions:** Depending on our results of prognostic markers of MM such as age, hemoglobin, albumin, and creatinine we feel confident to presume FLCe-MM as a subgroup with a worse prognosis. A decrease in PON1 and ARE activities may contribute to the prognosis and may be used as a prognostic tool in MM.

Keywords: Myeloma - free light chain - oxidative stress - paraoxonase - prognosis

Asian Pac J Cancer Prev, 15 (22), 9847-9851

Introduction

Multiple myeloma (MM) is a haematological cancer characterized by clonal proliferation of plasma cells. Monoclonal immunoglobulin (Ig) generation is observed in more than 80% of those affected. Each monoclonal Ig will include either a kappa light chain (LC) isotype or a lambda LC isotype. In the vast majority of the cases of monoclonal Ig in MM, there is an excess production of the relevant monoclonal LC, and in around 20% of the cases there is LC monoclonality with no detectable intact immunoglobulin. This detectable Ig LC is often known as "free light chain (FLC)" (Kyle et al., 2006; Stringer et al., 2011).

Serum FLCs are relatively freely filtered at the glomerulus due to their size [(22.5 kD for Kappa (κ) and 45 kD for Lambda (λ)] and their cationic net charge (Jarad et al., 2009). FLC reabsorption occurs in proximal tubular epithelial cells through receptors, and free LCs in tubular ultrafiltrate are received by receptors (megalin and cubilin), followed by endocytosis via the endosomal-

lysosomal pathway. Some FLC clones bind to megalin and some to cubilin (Batuman et al., 1990; Batuman et al., 1998; Sengul et al., 2003). As MM evolves, the amount of clonal FLC available in glomerular ultrafiltrate progressively increases, ultimately to the levels that overwhelm the reabsorptive capacity of proximal tubular epithelial cells. Therefore, increasing amounts of FLC are present in filtrate in the loop of Henle and in the distal tubule. There is a differential capacity of any given clone of FLC to aggregate with uromodulin (Tam-Horsfall protein) to form casts and finally obstruct tubular fluid flow (Sanders et al., 1998). Clinical manifestations of this phenomenon, known as "cast nephropathy" or "myeloma kidney", include acute kidney injury (AKI) and progressive renal failure.

Preliminary diagnosis of MM in about half of the patients will have AKI of a variable degree, and at least 10% will have severe AKI. The prognosis for patients with normal renal function is better than that for those with MM and AKI. This relates to the seriousness of AKI at time of diagnosis, such that those who require and receive

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dialysis treatment have a median survival of less than 12 months (Augustson et al., 2008; Dimopoulos et al., 2008).

Human serum paraoxonase (PON1) and arylesterase/lactonase (ARE) are lipophilic antioxidant enzymes. Serum PON1 binds to high density lipoprotein (HDL) and contributes to the elimination of organophosphorus compounds and free radicals. PON1 is one of the endogenous free-radical scavenging systems in the human organism (Aviram et al., 1998). Serum PON1 and ARE have been demonstrated to function as a single enzyme (Gan et al., 1991). Human serum PON1 indicates neither age-related change in activity nor gender differences (Geldmacher et al., 1983). Reduced PON1 enzyme activities have been showed in several groups of patients with hypercholesterolemia, diabetes mellitus, renal failure and cardiovascular disease, in which the patients are under increased oxidative stress (Mackness et al., 1991; Ayub et al., 1999; Yılmaz et al., 2012).

Human diseases are related with oxidative stress in one way or another. However, standardized measurement method that can measure the levels of oxidative stress is still not found. Recently, oxidant species or free radicals were monitored by determining total oxidant status (TOS) (Erel et al., 2005), and total antioxidant status (TAS) (Erel et al., 2004) as supposed to supply information on serum oxidative stress index (OSI) of an individual (Harma et al., 2003).

Thiols are endogenous compounds that include the sulfhydryl group (-SH) attached to a carbon atom. Both extracellular and intracellular redox states of thiols play a critical role in determining protein function and structure, regulation of enzymatic activity of transcription factors and antioxidant protection (Wlodek et al., 2002).

MM is commonly complicated by kidney injury with a major impact on long term outcomes. The aim of this study was to investigate the activity of serum PON1 and ARE in MM with and without FLC excretion, and to investigate possible alterations in oxidative stress.

Materials and Methods

A total forty MM patients (18 females and 22 males; average age: 67.55±8.39 years) who were hosted at Education and Research Hospital were prospectively included in the study. The patients were at various stages of disease, at different phases of treatment and of response.

Serum immunofixation electrophoresis and urine immunofixation electrophoresis were performed on patient samples using agarose gel via Helena Biosciences Europe Electrophoresis instrument. Urine samples were collected without using any preservative and stored at 4°C for less than 48 hours prior to analysis.

Patients with renal insufficiency were classified according to serum creatinine concentrations as; 23 patients with serum creatinine (sCr) levels in 0-1 mg/dl range, 9 patients with sCr levels 1.1-2 mg/dl range, and 8 patients with sCr levels >2 mg/dl. Glomerular filtration rate (GFR) was estimated using the Modification of Diet in Renal Disease (MDRD) formulae (Levey et al., 1999).

Blood samples were obtained after an overnight fasting state. Serum samples were then separated from the cells

by centrifugation at 3000 rpm for 10 minutes. Lipid parameters and other routine parameters were measured freshly. Remaining serum fractions were stored at -80°C and used to analyze PON1, ARE, TOS, TAS and total thiol concentrations.

Analytical methods

Measurement of paraoxonase and arylesterase enzyme activities in serum: PON1 and ARE enzyme activities were measured by using commercially available kits (Relassay®, Turkey). Fully automated PON1 activity measurement method consists of two different sequential reagents; the first reagent is an appropriate Tris buffer and it also contains calcium ion, which is a cofactor of PON1 enzyme. Linear increase of the absorbance of p-nitrophenol, produced from paraoxon, is followed at kinetic measurement mode. Non-enzymatic hydrolysis of paraoxon was subtracted from the total rate of hydrolysis. The molar absorptivity of p-nitrophenol is 18,290 M⁻¹ cm⁻¹ and one unit of paraoxonase activity is equal to 1 mol of paraoxon hydrolyzed per liter per minute at 37°C (Eckerson et al., 1983).

Phenylacetate was used as a substrate to measure the ARE activity. PON1, present in the sample, hydrolyses phenylacetate to its products, which are phenol and acetic acid. The produced phenol is colorimetrically measured via oxidative coupling with 4-aminoantipyrine and potassium ferricyanide. Non-enzymatic hydrolysis of phenyl acetate was subtracted from the total rate of hydrolysis. The molar absorptivity of colored complex is 4000M⁻¹ cm⁻¹ and one unit of arylesterase activity is equal to 1 mmol of phenylacetate hydrolyzed per liter per minute at 37°C (Haagen et al., 1992).

Measurements of the total oxidant and antioxidant status in serum: The TOS and TAS of the sera were analyzed by commercially available kits (Relassay®, Turkey) on an autoanalyzer (Architect® c16000, Abbott Diagnostics), using automated colorimetric measurement methods developed by Erel et al. (2005). In the TAS method, antioxidants in the sample reduce the dark blue-green colored 2, 2'-azino-bis (3 ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical to a colorless reduced ABTS form. The change of absorbance at 660 nm is related with the total antioxidant level of the sample. Using this method, the antioxidative effect of the sample against the potent free radical reactions initiated by the produced hydroxyl radical is measured (Erel et al., 2004).

In the TOS method, oxidants present in the sample oxidize the ferrous ion-chelator complex to ferric ion. The ferric ion makes a colored complex with chromogen in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample (Erel et al., 2005).

Oxidative stress index

The percentage ratio of TOS to TAS was accepted as oxidative stress index (OSI) (Harma et al., 2003). For calculation, the resulting micromolar unit of TAS was changed to millimoles per liter, and the OSI value was calculated according to the following formula: OSI

(arbitrary unit)=TOS (micromolar hydrogen peroxide equivalent per liter)/TAS (micromolar trolox equivalent per liter).

Measurement of thiol activity

Serum total thiol concentration or sulfhydryl groups (SH) were measured by the method originally described by Ellman et al. (1959) and modified by Hu et al. (1994). Briefly, 1 mL of buffer containing 0.1 M Tris, 10 mM EDTA (pH 8.2), and 50 μ L serum was added to cuvettes, followed by 50 μ L of 10 mM DTNB in methanol. Blanks were run for each sample as a test, but without DTNB. Following incubation for 15 min at room temperature (RT), sample absorbance was read at 412 nm on a spectrophotometer. Sample and reagent blanks comprised the substrate. The concentration of sulfhydryl groups was calculated using reduced glutathione as the free sulfhydryl group standard, and the results are expressed as millimoles. The CV for the measurement of the serum - SH level was 3.6%.

Routine parameters

The levels of triglycerides (TG), total cholesterol (TC), HDL-cholesterol (HDL-C), LDL-cholesterol (LDL-C), calcium (Ca), protein, albumin, creatinine, blood urea nitrogen (BUN) and uric acid were determined by using commercially available assay kits (Abbott) with an autoanalyzer (Architect[®] c16000, Abbott Diagnostics).

The levels of hemoglobin (HMG) were determined by using fully automated hematology analyzer (Sysmex[®] xt-2000i, Roche Diagnostics)

Statistical analysis

Statistical analyses were carried out using the statistical software version 11.5.1.0 (MedCalc[®], Mariakerke, Belgium). In abnormally distributed groups the results were presented with median and %95 confidence interval. The significance of the differences between groups was determined by Mann-Whitney U-test, and by Kruskal-Wallis test. Spearman correlation coefficient were used

to test the strength of any associations between different variables. P values less than 0.05 was accepted as the significance level.

Results

Serum immunofixation electrophoresis and urine immunofixation electrophoresis were performed on every patient, and the following paraproteins were determined: IgG kappa in 21 patients, IgG lambda in 4 patients, IgA kappa in 6 patients, IgA lambda in 2 patients, IgM kappa in 5 patients, and IgM lambda in 2 patients. FLC excretion detected in twenty-one MM patients (9 females and 12 males; average age: 67.02 \pm 9.89 years). The following FLC were determined: IgG kappa in 12 patients, kappa in 6 patients and lambda in 3 patients. FLC excretion was not detected in nineteen MM patients (9 females and 10 males; average age: 70.85 \pm 20.5 years).

FLCe-MM patients were older than NFLCe-MM patients (p=0.009). When laboratory findings were compared between two groups, it was found out that total protein, BUN, creatinin, and uric acid levels were significantly higher ((p=0.05), (p=0.001), (p=0.001) and (p=0.0022), respectively), while levels of hemoglobin and albumin were significantly lower in FLCe-MM patients ((p=0.009) and (p=0.04)). Overall demographic and laboratory findings are summarized in Table 1.

The anti-oxidant enzymes PON1 and ARE activities were significantly lower in patients with FLCe-MM, compared to those with NFLCe-MM (p=0.001 and p=0.008, respectively). T.thl and TAS were not significantly different (p=0.84 and p=0.14, respectively). The oxidant parameters (TOS and OSI) were higher in FLCe-MM, although did not reflect a significant difference (p=0.58 for TOS, p=0.80 for OSI) (Table 2).

TAS was lowest in sCr level 0-1 mg/dl group, followed by sCr>2 mg/dl group and highest in sCr 1.1-2 mg/dl group (p=0.02). Uric acid was lowest in sCr level 0-1 mg/dl group, followed by sCr level 1.1-2 mg/dl group and highest in sCr>2 mg/dl group (p=0.002) (Table 3).

Table 1. Laboratory Findings and Demographic Characteristics of FLCe-MM and NFLCe-MM

Parameter	FLCe-MM (n=21)	NFLCe-MM (n=19)	P
Age, mean \pm SD, years	72 (66-75)	64 (56-70)	0.009
Gender, male, n, (%)	12 (55%)	10 (52.5%)	NS
Total protein, g/dl	9.6 (7.7-12.1)	7.8 (7.4-10)	0.04
Albumin, g/dl	2.9 (2.4-4)	3.8 (3.5-4.2)	0.04
Hemoglobin, g/dl	8 (7.4-9.4)	10 (8.5-11)	0.009
Calcium, mg/dl	9.4 (8.5-11)	9.5 (9-9.7)	NS
BUN, mg/dl	23 (21-35)	16 (11-20)	0.001
Creatinin, mg/dl	1.2 (0.8-3.5)	0.8 (0.7-1.1)	0.001
Uric acid, mg/dl	7.3 (5.7-10.1)	5.3 (4.5-7.4)	0.022
GFR, mL/min/1.73 m ²	51 (17.9-88)	90 (58.6-103.8)	0.02
TC (mg/dl)	128 (92-179)	170 (134-207)	0.03
TG (mg/dl)	126 (67-170)	180 (69-152)	NS
LDL (mg/dl)	69 (41-103)	97 (72-140)	0.01
HDL (mg/dl)	30 (20-41)	38 (33-42)	NS

*In the patients with FLCe-MM, albumin and hemoglobin were significantly reduced, whereas the age, total protein, BUN, creatinin, and uric acid levels had significantly increased when compared with those of NFLCe-MM. (GFR: Glomerular filtration rate was estimated using MDRD formulae, NS: Non significant, Median (95% CI for median) for all parameters)

Table 2. PON1 and ARE Activities were Significantly Decreased in FLC-MM. T.thl, TAS, TOS and OSI Levels were not Statistically Significant

Parameter	FLCe-MM	NFLCe-MM	P
PON1(U/L)	84 (58-117)	178 (120.1-257.4)	0.001*
ARE(kU/L)	105 (80-212.7)	226 (167.3-295)	0.008*
T.thl (mm/L)	1350 (862-1742)	1250 (925-1721)	0.84
TAS(nmol Trolox/L)	3.1 (2.7-3.4)	2.9 (2.6-3.09)	0.14
TOS(μ molH ₂ O ₂ Equiv./L)	11.2 (3.5-15.5)	6 (3.21-16.4)	0.58
OSI	0.34 (0.13-0.54)	0.19 (0.09-0.67)	0.80

*Statistically significant and Median (%95 CI for median) for all parameters

Table 3. Statistically Significant Differences were Obtained in TAS and Uric acid, being the Lowest in sCr Levels 0-1 Range. Average for all parameters

sCr (n)	PON	ARE	t.thiol	TAS	TOS	OSI	Uric A.
0-1 (23)	21.5	18.7	23.5	16.2	17.8	18.5	15.3
1.1-2 (9)	20.5	27.6	14.2	27.4	23	21.7	24.3
>2 (8)	17.4	17.4	19	25.1	25.2	25	31.2
P	0.1	0.69	0.11	0.02	0.23	0.37	0.002

The correlation between TAS levels and uric acid levels and GFR were significant ($r=0.395$ $p=0.001$, $r=-0.43$ $p=0.005$, respectively). The correlation between uric acid levels and GFR with PON1, ARE, T. thiol, TOS, OSI were not significant.

Discussion

With traditional chemotherapy, the average life span of patients with MM has ranged from two or three years for patients >65 years to five or six years for younger patients. Age is a well established good performance status and younger age is a favorable prognostic feature. Other well known prognostic factors in MM are: increased haemoglobin levels, renal function impairment, and low serum albumin (Durie et al., 1975; Greipp et al., 2005; Blade et al., 2010). Demographic and laboratory findings obtained in this study, demonstrated significantly lower albumin and hemoglobin levels; whereas significantly higher age, BUN, sCr and uric acid levels in FLCe-MM patients compared to NFLCe-MM patients. These results showed that our NFLCe-MM group was with favourable prognostic features compared to FLCe-MM group. Our study also demonstrated a significant decrease in PON1 and ARE activities in FLCe-MM patients when compared to those in NFLCe-MM group, whereas T.thl, TAS, TOS and OSI levels were not significantly different.

There is strong evidence that show a severe proinflammatory and cytotoxic potential of monoclonal free light chain. There exists a differential capacity of any given clone of FLC to activate proximal tubular epithelial cells to produce proinflammatory cytokines through activation of NF κ B. This process may contribute to the inflammatory cell infiltration and accelerated fibrosis that is seen in cast nephropathy (Sengul et al., 2003; Stringer et al., 2011). Another factor of the pathogenicity of monoclonal FLC towards proximal tubular epithelial cells is mediated through the formation of hydrogen peroxide (H_2O_2). *In vitro* studies showed that this was generated after FLC endocytosis and indicated a high level of oxidative stress (Morigi et al., 2002). It has subsequently been established that H_2O_2 production by monoclonal FLC mediated the oxidation and activation of c-Src (a redox sensitive, non-receptor tyrosine kinase) (Basnayake et al., 2010). These and other studies show that monoclonal FLCs have a greater inflammatory effect on proximal tubular epithelial cells than other freely filtered proteins (Li et al., 2008).

Human serum paraoxonase (PON1) and arylesterase/lactonase (ARE) are the enzymes that have lipophilic antioxidant characteristics. There is evidence in some studies that PON1's antioxidant function begins at the level of lipoprotein (LDL and HDL) protection against oxidative alteration by ROS. These enzymes also reduces lipid hydroperoxides to hydroxides and presents a peroxidase-like activity, as PON1 was demonstrated to degrade hydrogen peroxide (H_2O_2), a major reactive oxygen species produced under oxidative stress (Yilmaz et al., 2012). The studies of Sharma et al. (2009) indicate significant changes in antioxidant defense system in MM

patients, which may lead to enhanced action of oxygen radicals, resulting in lipid peroxidation (Sharma et al., 2009). These results suggest that oxidative stress exceed antioxidant defense effects protein structures such as antioxidant enzymes. Impaired oxidant/antioxidant balance and increased H_2O_2 have disrupted the structure of PON-ARE in FLCe-MM. However, cachexia and malnutrition in cancer patients are important problems due to a variety of mechanisms. In the later stages of disease, malnutrition and inflammation suppress protein synthesis. PON1 and ARE activities may decrease due to suppressed protein synthesis, cachexia and malnutrition, as the host response to the tumor (von Meyenfeldt et al., 2005; Ellidag et al., 2013).

In our study, T.thl, TAS, TOS and OSI levels were not significantly different in FLCe-MM when compared to NFLCe-MM. Interestingly, TAS and uric acid levels were significantly higher in patients with increased serum creatinin levels and there was a positive correlation between TAS and uric acid levels. Uric acid is an effective antioxidant. However, it should be kept in mind that uric acid is a molecule with both beneficial and harmful effects on human health. Epidemiological and clinical evidence strongly suggests that hyperuricaemia is a risk factor for coronary artery disease, where enhanced oxidative stress plays an important pathophysiological role. It is thus conceivable that under these conditions its antioxidant activity is overcome by the pro-oxidant and proinflammatory effects of ROS accumulation (Strazzullo et al., 2007). Some researchers found that uric acid had detrimental impact on the cardiovascular system, such as mediating the immune response upon a possible cell injury, increasing the endotoxin stimulated tumor necrosis factor-alpha production and hence the proinflammatory immune activation (Netea et al., 1997; Shi et al., 2003). Promotion of low-density lipoprotein oxidation, smooth muscle cell proliferation and platelet activation and adhesion by uric acid has been demonstrated *in vitro* (Lippi et al., 2008).

In conclusion, we found prognostic markers of MM such as age, hemoglobin, albumin, and creatinine parameters to be in favor of NFLCe-MM; thus we feel confident to presume FLCe-MM as a subgroup with a worse prognosis.

Our findings suggest FLC excretion in MM to become an useful prognostic marker. During the routine follow-up of these patients, amount of FLC excretion must be a warning for the development of multiple myeloma in the kidney. We detected lower activities of the antioxidant enzymes PON1 and ARE in patients with FLCe-MM, in line with previous *in vitro* studies showing that myeloma kidney was generated after FLC endocytosis and it indicated a high level of oxidative stress (Netea et al., 1997). Decreased PON1 and ARE activities may contribute to worse prognosis and development of comorbid disorders. Although PON1 and ARE enzyme activities were decreased in FLCe-MM, the oxidant/antioxidant balance was surprisingly conserved. This was probably due to the increased uric acid levels in FLCe-MM.

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