An association between manganese superoxide dismutase polymorphism and outcome of chemotherapy in acute myeloid leukemia

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Manganese superoxide dismutase (MnSOD) protects cells against oxidative stress by eliminating superoxides. Hypothetically, decreased MnSOD levels in cancer might lead to increased oxidative stress and, thus, to increased sensitivity of cells to chemotherapy agents. Eighty-nine patients with acute myeloid leukemia (AML) were analyzed for a functional C to T polymorphism of *MnSOD*, which could potentially lead to decreased enzyme concentrations inside mitochondria. A significant survival advantage (p=0.02) was observed for those AML patients carrying T-containing alleles of *MnSOD* compared to the patients with the CC genotype. These preliminary results may indicate an important role for genetic factors regulating the cellular redox state in determining the outcome of leukemia chemotherapy.

Key words: MnSOD, GPX, catalase, polymorphism, AML, survival.

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Oxidative stress-induced apoptotic cell death is an important mechanism involved in the effects of chemotherapy agents.[1] A battery of antioxidant enzymes, however, protects cells from oxidative stress. Not surprisingly, elevated antioxidative capacity of cancer cells has been found to be associated with drug resistance.[2] Hypothetically, drug sensitivity might be regulated by functional polymorphisms in the genes coding for antioxidative enzymes. The published C to T polymorphisms in codon 16 of the mitochondrial targeting sequence of manganese superoxide (*MnSOD*),[3] in codon -262 in the promoter region of catalase (*CAT*)[4] and in codon 198 in exon 1 of the glutathione peroxidase (*GPX*) type I gene[5] potentially lead to variations in cellular enzyme activities. The alanine-containing MnSOD (as a result of C in codon 16) is targeted into mitochondria, while the valine-containing MnSOD (as a result of T in codon 16) is retained outside mitochondria.[6] In the case of *CAT*, individuals homozygous for TT or heterozygous for CT have higher CAT concentrations in blood compared to individuals homozygous for CC.[4] On the other hand, the presence of T instead of C in codon 198 of *GPX* seems to lead to decreased enzyme activity in the cells.[5] Decreased MnSOD could be expected to lead to increased superoxide concentrations inside mitochondria, while decreases in CAT and GPX cause increased cellular hydrogen peroxide levels. In the present study we examined the hypothesis that the described polymorphisms in *MnSOD*, *CAT* and *GPX* genes might be related to the outcome of patients with acute myeloid leukemia (AML).

Design and Methods

Patients and samples

The study was conducted in Oulu University Hospital under a protocol approved by the Ethics Committee of the hospital. Informed consent was provided according to the Declaration of Helsinki. The patients participating in the present study were treated according to the uniform national AML protocols agreed by the Finnish Leukemia Group.[7-9] The diagnosis of AML was based on standard cytological criteria according to the French-British-American (FAB) classification and immunophenotype as assessed by flow cytometry using standard lineage markers. At diagnosis, bone marrow and blood were examined for cytogenetic abnormalities with standard banding techniques and fluorescence *in situ* hybridization analysis. Peripheral blood samples for the *MnSOD*, *CAT* and *GPX* genotype analyses were obtained at diagnosis from a total of 89 patients between 1988 and 2000. The clinical and laboratory data of the patients are shown in Table 1.

Identification of the MnSOD, CAT and GPX gene polymorphisms

Genotyping was done by polymerase chain reaction (PCR) as described in references. PCR was done for *MnSOD*[10] using primers 5'-AGCCCAGCCGTGCGTAGAC-3' and 5'-TACTTC-TCCTCGGTGACG-3', for *CAT*[4] using primers 5'-TAAGAGCTGAGAAAGCATAGCT-3' and 5'- AGAGCCTCGCCCCGCCGGACCG-3', and for *GPX*[11] using primers 5'-TGTGCCCCTACGCAGGTACA-3' and 5'-CCCCCGAGACAGCAGCA-3'. Analyses were successful for all of the 89 patients. About 10% of the samples were analyzed twice with 100% concordant results as a guarantee of laboratory quality control.

Statistical analysis

The SPSS 12.0 for Windows (Chicago, IL, USA) software package was used for statistical analyses. Survival estimates were obtained using the Kaplan-Meier method and calculated from the date of diagnosis to the date of death

Table 1. Clinical and laboratory characteristics of the study patients with acute myeloid leukemia at diagnosis (n=89).

Age (y), median (range)	53 (17 - 86)
Gender, N (%)	
Female	42 (47)
Male	47 (53)
Protocol, N (%)	
AML-86	10 (11.2)
AML-92	53 (59.6)
Other	26 (29.2)
FAB, N (%)	
M0	5 (5.6)
M1	19 (21.3)
M2	25 (28.1)
M3	4 (4.5)
M4	26 (29.2)
M5	7 (7.9)
M6	3 (3.4)
M7	0 (0)
Karyotype, N (%)	
Favorable*	3 (3.4)
Intermediate°	64 (71.9)
Unfavorable#	22 (24.7)

*FAB:French-American-British classification, N: number of patients. *t(8;21) ± -Y, t(15;17), inv(16)/t(16;16); °normal karyotype; all other changes not belonging to either the favorable or unfavorable group including three karyotypes that could not be analyzed. #-5/5q-, -7/7q-, t(9;22), t(6;9), 11q,20q,21q,3q,17p-, complex karyotypes with ≥3 changes.*

or to the most recent follow-up information at the end of December 2004. A Cox regression model was used to assess the effects of the gene polymorphisms and other potential prognostic factors, including age, gender, FAB group, treatment protocol and pretreatment karyotype, on survival. The patients were divided into three prognostic groups according to cytogenetics. The favorable cytogenetic group included t(15;17), t(8;21) in the presence or absence of del Y, inv(16) or t(16;16). The unfavorable group contained aberrations of chromosomes 5 or 7, aberrations of 3q, 11q, 17p, 20q or 21q, t(6;9), t(9;22), or a complex karyotype with e 3 changes. All other karyotype changes as well as normal karyotype and AML with no analyzable karyotype (three cases) were categorized into the intermediate group.

Results and Discussion

Overall survival and risk of death according to the various diagnosis-phase characteristics

The pre-treatment karyotype showed the strongest correlation with overall survival (Figure 1A). In patients with the favorable karyotype (n=3), the median overall survival was not reached, while in the intermediate group (n= 64) it was 38 months (95% CI: 17.6-58.4) and in the group with unfavorable cytogenetics (n=22) it was 10 months (95%CI: 4.5-15.5). The patients with unfavorable karyotypes were 2.8 (95% CI: 1.6-4.9) times more likely to die than were patients with the intermediate karyotype ($p<0.001$). Older age and non-intensive treatment protocol also correlated with poorer outcome.

Overall survival and genotype distributions of MnSOD, CAT and GPX

The median overall survivals for patients with *MnSOD* genotypes CC, CT and TT were 6 months (95%CI: 0.5-16.0), 36 months (95% CI: 19.6-52.4, $p=0.02$) and 20 months (95% CI: 0.5-39.5, p=NS), respectively. As Figure 1B shows, overall survival was significantly ($p=0.02$) longer for the patients with T-containing genotypes (n=69) than for the patients with the CC genotype (n=20) of *MnSOD*. When overall survival was analyzed according to the treatment protocol, it was still found that T-containing genotypes conferred an advantage for overall survival, but that this was statistically significant only among patients in the AML-92 protocol (Figure 1C). The median overall survivals for the patients with *CAT* genotypes TT, CT and CC were 26 months (95% CI: 0.5-61.0), 24 months (95% CI: 0.5-53.9) and 26 months (95% CI: 12.0-40.0), respectively. There was no difference in overall survival between patients with the C-containing genotypes and patients with the TT genotype. The median overall survivals for the patients with *GPX* genotypes CC, CT and TT were 13 months (95%CI: 0.5-35.4), 36 months (95% CI: 18.9-53.1) and 20 months (95% CI: 5.9-34.1), respectively. There was no difference in overall survival between patients with a T-containing genotype and patients with the CC genotype.

Overall survival and gentotype distributions of MnSOD in various karyotype groups

The favorable karyotype group comprised only three patients, thus the comparison of overall survival between patients with the CC genotype and T-containing genotypes is impossible. Interestingly, however, one of the patients carried the CC genotype of *MnSOD*, and died 3 months after diagnosis, while the other two patients with T-containing genotypes of *MnSOD* are still alive, one 137 months and the other 161 months after diagnosis. In the intermediate karyotype group, the median overall survival for the patients with the CC genotype of *MnSOD* (n=11) was 42 months (95%CI: 0-84.0) while that for the patients with the T-containing genotype (n=53) was 38 months (95%CI: 1.0-75.0, p=NS). In the unfavorable karyotype group, on the other hand, the median overall survival was only 3 months (95%CI: 0.2-5.8) for the patients with the CC genotype of *MnSOD* (n=8), while for the patients with T-containing genotypes (n=14) it was significantly longer: 11 months (95%CI: 7.3-14.7, $p=0.002$).

Risk of death and genotype distributions of MnSOD, CAT and GPX

Patients with the CC genotype of *MnSOD* were 2.2 (95% CI: 1.0-4.8) and 1.9 (95% CI: 1.1-3.5) times more likely to die than were patients with the TT genotype or patients with the CT genotype, respectively, while neither *CAT* nor *GPX* genotypes had any effect on risk of death, after overall survival had been adjusted for other significant prognostic factors, such as age, pre-treatment karyotype and treatment protocol (Table 2). The present study describes the association between the *MnSOD* C16T polymorphism and survival after chemotherapy for AML. Patients homozygous for the alanine-containing allele (CC genotype) had a significantly worse outcome than the patients who were either heterozygous (CT genotype) or homozygous (TT genotype) for the valine-containing allele. This finding highlights the potential importance of mitochondria-formed superoxides in chemotherapy-induced cytotoxicity and in the elimination of leukemia.

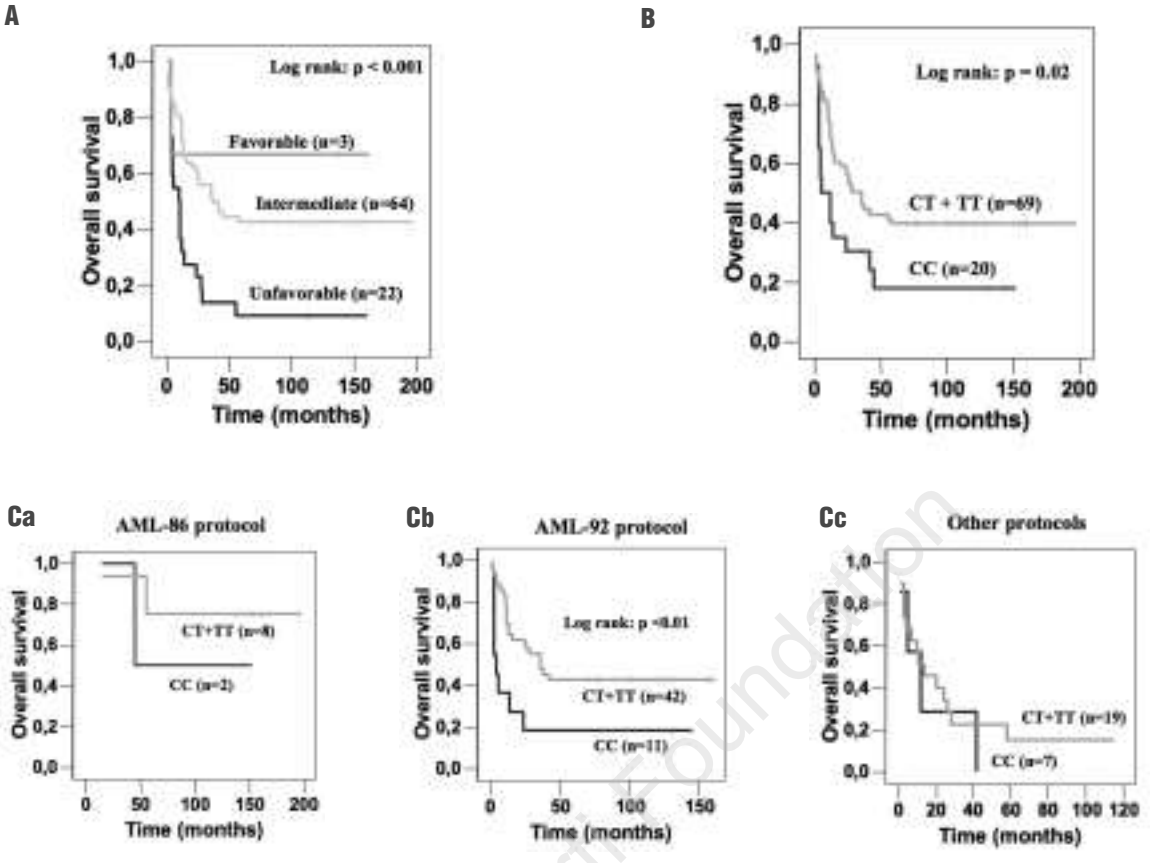

Figure 1. **Overall survival of patients with AML by cytogenetics** (A), **by *MnSOD* polymorphisms** (B), **and by *MnSOD* polymorphisms in patients treated with different protocols** (C).

Table 2. **Association between manganese superoxide dismutase (*MnSOD*), catalase (*CAT*) and glutathione peroxidase (*GPX*) genetic polymorphisms and survival after treatment for acute myeloid leukemia in a Cox regression model.**

Genotype	*Cases*	*Risk of death (95%CI)*	*p*	*Risk of death (95%CI)**	*p*
MnSOD	89				
TT	20	1	1		
CT	49	0.8 (0.4 - 1.6)	0.60	1.2 (0.6 - 2.5)	0.57
CC	20	1.8 (0.8 - 3.8)	0.14	2.2 (1.0 - 4.8)	0.05
CC+CT	69	1.0 (0.5 - 2.0)	0.92	1.5 (0.7 - 2.9)	0.24
CT+TT	69	1	1		
CC	20	2.0 (1.1 - 3.5)	0.03	1.9 (1.1 - 3.5)	0.03
CAT	89				
CC	38	1	1		
CT	39	0.8 (0.5 - 1.5)	0.50	0.7 (0.4 - 1.3)	0.28
TT	12	0.8 (0.3 - 1.9)	0.58	0.9 (0.4 - 2.4)	0.85
CT+TT	51	0.9 (0.5 - 1.5)	0.67	0.8 (0.5 - 1.5)	0.54
CC+CT	77	1			1
TT	12	0.9 (0.4 - 2.1)	0.82	1.1 (0.5 - 2.6)	0.82
GPX	89				
TT	29	1	1		
CT	39	0.8 (0.4 -1.5)	0.44	0.6 (0.3 - 1.1)	0.11
CC	21	1.0 (0.5 - 2.0)	0.98	0.8 (0.4 - 1.6)	0.51
CC+CT	60	0.9 (0.5 -1.5)	0.64	0.7 (0.4 - 1.2)	0.21
CT+TT	68	1			1
CC	21	1.11 (0.6 - 2.1)	0.75	0.9 (0.5 - 1.8)	0.82

**Risk of death for adjusted model: age at diagnosis, pretreatment karyotype and treatment protocol. CI: confidence interval.*

More generally, it may also indicate that genetic factors regulating the cellular redox state are crucial in determining the outcome of leukemia chemotherapy. The most commonly used chemotherapy agents in AML induce oxidative stress and lead to mitochondrial damage and apoptotic cell death.[1,12] Thus, a decreased capability of the mitochondria to handle oxygen stress may be translated into increased drug-sensitivity, and *vice versa*, a high capacity may protect cells from toxicity and lead to the occurrence of drug resistance and the presence of minimal residual disease, and ultimately, to relapse and decreased survival. MnSOD is the only enzyme capable of detoxifying superoxides inside the mitochondria. Superoxide, if not properly destroyed there, can inhibit and damage mitochondrial function either by reacting with nitric oxide or by inactivating the Fe-S centers in the electron transport chain.[13] MnSOD is synthesized in the cytoplasm as a precursor protein and is transported by the mitochondrial targeting sequences into the mitochondria, where it is processed into an active homotetramer.[13,14] A common C to T polymorphism in the mitochondrial targeting sequences of the *MnSOD* gene leads to formation of a valine-containing protein instead of the normal alanine-containing protein.[3] Experimentally, this valine form of the enzyme is less active and unable to translocate into the mitochondrial matrix.[6] Thus, it is plausible to argue that cancer patients carrying the valine-containing protein should be more sen-

sitive to cytotoxic drugs. According to the present study, this does seem to be the case in AML.

An active MnSOD generates hydrogen peroxide, which must be eliminated either inside or outside mitochondria, since elevated levels of this peroxide will also inhibit cell proliferation and induce cell death. The extent of this elevation is related to the levels of peroxide-removing enzyme activities.[1] In the present study, the functional polymorphisms of *CAT* and *GPX* did not seem to have any influence on survival, which argues against our hypothesis. There are three different enzyme systems in the cell involved in the elimination of hydrogen peroxide.[15,16] Of these enzymes, GPX and peroxiredoxins, but not CAT, are also found in the mitochondria.[16-18] Hydrogen peroxide, however, is able to diffuse out from the mitochondria, where all three enzymes can eliminate it. Thus, it is perhaps less plausible that the described polymorphisms of *CAT* and *GPX* could lead to such depressed concentrations of these enzymes to significantly impair the elimination of hydrogen peroxide. Moreover, the findings of the present study seem to point to a more important role for superoxide than hydrogen peroxide in the transmission of chemotherapy-induced cytotoxicity for the elimination of AML.

Pretreatment karyotype has been observed to be an important prognostic factor in AML,[19] as was also noted in the present study. However, *MnSOD* genotyping might well complement diagnostic cytogenetics and thus provide a more accurate risk assessment, especially among patients with an unfavorable karyotype. This may ultimately enable a more sophisticated treatment approach in AML. It should be noted that although the association between MnSOD and chemosensitivity of various cancer cell lines has been shown previously,[20] the results of the present study must be considered as preliminary since the number of patients studied was quite small and the patients were not consecutive patients in a single study. A larger cohort of patients needs to be investigated before proper evaluation can be made of the significance of polymorphisms of *MnSOD* mitochondrial targeting sequences on the outcome of AML.

All authors (PK, SR, MS, SK, PS, TS, MJS, VK and E-RS) made substantial contributions to the conception and design of the study, analysis and interpretation of data and revising the arcticle, and provided final approval of the present version of the manuscript. In particular, SR performed the PCR analysis. PK is the corresponding author and is responsible of all the draft versions of the article. There is no overlapping publication. The authors declare that they have no potential conflicts of interest.

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