The 5alpha-Reductase Type II A49T and V89L High-Activity Allelic Variants are More Common in Men with Prostate Cancer Compared with the General Population.

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The 5α-reductase type II A49T and V89L high-activity allelic variants are more common in men with prostate cancer compared with the general population

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Disclosure Statement

None of the authors have financial and/or personal relationships with people or organizations that could inappropriately influence (bias) their work. The corresponding author had full access to all data in the study and had the final responsibility for the decision to submit the manuscript for publication.

Abstract

Objectives

To compare men with prostate disease with those from the general population regarding polymorphisms in the androgen receptor gene and in the 5α-reductase II (SRD5A2) gene.

Materials and Methods

The SRD5A2 polymorphisms A49T, V89L and R227Q, the androgen receptor CAG and GGN repeats and sex hormone status was investigated in men with prostate cancer (CaP) (n=89), benign prostate hyperplasia (n=45) and healthy military conscripts (n=223).

Results

The SRD5A2 high-activity allele variants A49T AT and V89L LL were more frequent in CaP-patients compared to general population, p=0.026 and p=0.05, respectively. CaP progression was, however, independent of SRD5A2 variants. In contrary, men with GGN<23 had a higher risk of dying from the disease than their counterparts with longer repeats.

Conclusions

Men with CaP were more often genetically predisposed to a higher enzymatic activity in the turn over from T to DHT compared to the general population. In our population, androgen receptor genotype affected CaP outcome.

Introduction

In Northern Europe prostate cancer CaP has taken number one position as the leading cause of cancer-related death in men [1]. Despite its high incidence, the etiology and mechanisms underlying development and progression of CaP are poorly understood. It has been hypothesized that CaP is a manifestation of an excessive response to androgens or an increased intraprostatic androgen metabolism [2]. This could be a net-result of either increased serum testosterone levels, higher rate of conversion of testosterone to 5α-dihydrotestosterone (DHT) or variation in androgen receptor (AR) sensitivity.

The enzyme 5α-reductase (SRD5A) catalyses the conversion of testosterone to DHT. The presence of two isoenzymes have been identified, each showing a tissue specific expression pattern. Type I is expressed in liver and non-genital skin after birth, whereas type II is expressed in the prostate, Wolffian ducts and genital skin throughout life [3].

A higher SRD5A2 activity in African Americans than in Asians has been observed, which parallels observed ethnic differences in CaP risk [4]. The SRD5A2 gene contains three polymorphisms: a valine to leucine substitution at codon 89 (V89L), an alanine 49 to threonine transversion (A49T) and arginine to glutamine at codon 227 (R227Q). The A49T mutation seemed to have the highest impact on the enzymatic activity [5] increasing the conversion of testosterone to DHT 6-fold *in vitro* [6], whereas the V89L L-allele was associated with approximately 30% lower SRD5A2 activity [7] and lower prevalence in African Americans [8]. The R227Q mutation was first reported in boys with hypospadias and micropenis [9]. Recently a study from China, a low risk country for CaP, taking all three variants into consideration, could not confirm a significant association of these SRD5A2 polymorphisms with CaP risk [10]. However, a small risk of these markers could not be ruled out because of the rarity of certain marker genotypes in the investigated population.

Many studies have demonstrated a role of androgens in prostate growth and differentiation and androgen ablation is a one of the first treatments for patients with CaP. It has however recently been a matter of debate whether the androgen independent aggressive type of disease that occurs over time could be a consequence of the withdrawal of androgens [11].

Both testosterone and DHT act through the AR. There is only one AR gene located at Xq11-12 [12], but there are multiple allelic variants of the AR gene in the general population[13] due to two polymorphic trinucleotide repeats of CAG and GGN, coding for glutamine and glycine, respectively. Each polymorphism is varying in length among individuals in the general population and both the CAG and the GGN repeats have been found to regulate AR activity *in vitro* [14,15]. In particular the CAG segment has been extensively investigated with respect to CaP and some studies have suggested that shorter repeats might confer a higher risk of CaP [16,17], but no firm conclusions regarding any of the repeats could yet be drawn.

Very little is currently known about the associations between SRD5A2 and AR genetic variants and androgen concentrations in the circulation in men with CaP compared to healthy individuals. In most studies to date, men with benign prostate hyperplasia (BPH) have been used as controls, although prostate pathology is present in this category of men. The aim of our study was therefore to study whether these factors play a role for the risk and progression of CaP compared to men with benign prostate disease and healthy men from the general population.

Materials and methods

Subjects

The study population consisted of 134 Swedish men undergoing ultrasound-guided biopsies of the prostate on suspicion of prostate cancer, due to serum PSA concentration ≥4 ng/ml. The men were born 1910-1952 and their median age at time of biopsy was 67 years. CaP was confirmed in 89 patients, whereas 45 had benign prostate hyperplasia (BPH). All stages and grades of CaP were included (Table 1). In men with CaP, the mean age at diagnosis was 69.3, whereas men with BPH on average were 64.5 years, which was statistically significantly lower (p=0.002). During xx months of follow-up, 16 men (18%) with CaP died as a consequence of the disease.

Three hundred and five men under compulsory medical examination for military service, who accepted to take part in a study on reproductive function were used as a reference group [18]. This group can be considered as representative for the general adolescent male population in southern Sweden.

In order to exclude any impact of ethnic variation, genotyping was performed only in men of Swedish origin (n=223). All men participated with written informed consent according to protocols approved by the ethical review board of Lund University.

Hormone analysis

Sex hormone binding globulin (SHBG), testosterone and Luteinizing Hormone (LH) levels in blood were measured at Malmö University Hospital, Malmö, Sweden. Testosterone levels were measured using an immunoassay (Access®; Beckman Coulter Inc., USA). Laboratory total assay variation was 2.8% at 2.9 nmol/l and 3.2% at 8.1 nmol/l. Plasma FSH and LH concentrations were measured by means of immunoassays (Immuno 1®; Bayer Diagnostics Division, USA). Laboratory total assay variation for FSH was 2.5% at 2.9 IU/l and 1.4% at 15

IU/l, and for LH it was 2.6% at 3.0 IU/l and 1.7% at 15 IU/l. Serum sex hormone-binding globulin (SHBG) was measured using an immunoassay (Immulite® 2000; Diagnostic Products Corp., USA). Total assay variation was 3.7% at 29 nmol/l and 6.7% at 85 nmol/l.
Free testosterone was calculated based on known concentrations of testosterone and SHBG.

Genetic analyses

Genomic DNA was extracted from peripheral leukocytes by standard methods. Analysis of AR gene polymorphism was performed according to Lundin *et al* [19].
Allele-specific PCR was performed to detect the A49T, V89L and R227Q variants in the SRD5A2 gene. In order to determine the A49T and the V89L polymorphisms, exon 1 of the SRD5A2 gene was first amplified with the flanking primers X1f (5'-TGG GAG GCA GGA TGG AGG-3') and X1r (5'-CGC CGG GAG CAG GGC AGT-3') (Invitrogen, Edinburgh, Scotland) at concentrations of 0.5 μM. Each 50μL reaction was done using 50 pg DNA, 45mM KCl, 10 mM Tris-HCl (pH 8.4 at 70˚C), 0,1 % Tween 20, 1,50 mM $MgCl_2$, 0,2 mM dNTP (Roche Diagnostics, Bromma, Sweden), and 1.0 units of Dynazyme DNA polymerase (Finnzymes Oy, Espoo, Finland). Amplifications were carried out for 40 cycles; including denaturation for 1 min at 96°C, primer annealing for 60 sec at 60°C and primer extension for 3 min at 72°C, with an initial denaturation step for 3 min at 96°C, and a final extension for 7 min at 72°C.
In a subsequent allele-specific nested PCR two reactions for each subject was done, each containing one mutant or one wild-type specific primer, together with an upstream and a downstream primer. PCR-conditions were established to generate both a short allele-specific and a longer control band in the presence of the variant, and only the longer control band in its absence. The nested reactions were carried out as before, but with the two primers X1fn (5'-ATG GAG GGG CGG GAG CCA-3') and XIrn (5'-AGG GCA GTG CGC TGC ACT-3') at

concentrations of 0,2 µM. Regarding the A49T polymorphism, the reaction was carried out with 0,5 µM of either the allele-specific primer A49 (5'-AAC CAG GCG GCG CGG GC-3') or T49 (5'-AAC CAG GCG GCG CGG GT-3'). The cycles-conditions were as described above.

To detect the V89L base substitution 0,025µM of the downstream primer X1fn, 0,05µM of the non-specific upstream primer X1rn and 0,6 µM of either the allele-specific primer V89 (5'-ACC TGT GGA AGT AAT GTA C-3') or L89 (5'-ACC TGT GGA AGT AAT GTA G-3') was used.

The same strategy was used regarding the R227Q variant, with the primers X4fn (5'-ATT CAG TTG CAA TGA TTG ACC TT-3') and 2x-4r (5'-TCT GCG GGT TAA AAG CCT GTT-3') used in the first reaction and 0,5 µM of the nesting primers X4fn, X2-4r (5'-AGA AGA AAG CTA CGT GAA TGC T-3') and one of either the allele-specific primer R227 (5'-CTA TGG TGG TGA AAA GCT C-3') or Q227 (5'-CTA TGG TGG TGA AAA GCT T-3'). To verify the results, sequencing of samples representing each genotype was performed using the Big Dye Primer Cycle Sequencing Ready Reaction Kit and the ABI Prism310 DNA sequencer (PE Corporation, Foster City, CA, USA).

Statistical analysis

Correlations between numeric variables were evaluated by Spearman's correlation test. Correlations between nominal parameters were tested with the Chi-square test. The Mann-Whitney test was used to compare numeric variables in different genotypes. By applying a Cox multivariate regression model we evaluated the most significant parameters as prognostic markers for death from prostate cancer. Values of $p < 0,05$ were considered significant.

Results

Hormone analysis

In men with CaP or BPH, the AR polymorphisms did not correlate to serum hormone levels of testosterone, SHBG or LH. The concentration of free testosterone, calculated as T/SHBG increased with GGN length (Fig 1), which was not seen for the CAG-repeat. Serum levels for testosterone, SHBG and LH were equal in men with different genotypes in the SRD5A2 gene, though men with the AT genotype had higher LH-levels in (Table 2).

AR polymorphisms

In the whole study population, the AR gene was ranging from 14 to 32 repeats. The average CAG length among men diagnosed with CaP was 22.0, not differing from those with BPH, who on average had 21.6 ($p=0.41$) or conscripts with 21.9 on average ($p=0.86$). Neither was there any difference between men with BPH and conscripts ($p=0.479$). CAG≤17 was found in 3.1% of the conscripts compared to 5.6% and 6.7% in CaP and BPH groups, respectively ($p=0.42$).

Among the conscripts the GGN=23 was the dominating allele, comprising 52% (n=116) of the whole study population (n=223). The frequency of GGN=24 was 32% (n=71), whereas 14% (n=32) had GGN<23 and 2% (n=4) GGN>24.

In total 90% (n=80) of all men with diagnosed CaP had GGN lengths of either 23 (54%, n=48) or 24 (36%, n=32) GGN. Only 10.0% (n=9) had GGN<23. In the BPH group also 89% of the men carried either 23 (69%, n=31) or 24 (20%, n=9) GGN, whereas 11% (n=5) had shorter repeats. Statistically significant differences were neither found between men with CaP and BPH nor between CaP and conscripts. No particular combinations of CAG/GGN lengths were found to differ between men in the two study groups and the conscripts.

SRD5A2 polymorphisms

Among men with prostate cancer, the mean age for those carrying the SRD5A2 A49T AA genotype was 69.2 years, whereas those with the AT genotype were 71.5 years (p=0.48). The mean age among men with BPH and AA genotype was 65.0 years at time for diagnosis, whereas those with the AT genotype were 60.6 years. In Table 3 allele frequencies of each of the three markers are shown, by case-control status. The high activity AT variant was more frequent among CaP patients compared to the general population but not compared to patients with BPH. In men with CaP, the AR CAG repeat length in the AT group was not statistically different from the AA group, neither was there any correlation to GGN-length, median=23 in both groups.

The low activity homozygote V89L LL allele was less common in patients with CaP compared to the general population but not compared with BPH patients. The heterozygous VL was equally common among patients as among conscripts.

The Q of the R227Q marker was absent both among cases and controls.

Progression

Regarding the AR CAG polymorphism, no differences were seen in mean CAG-repeat lengths for patients with or without metastasis at diagnosis, or whether they died from CaP or not. Four out of 9 men with GGN<23 (44%) died from CaP during follow-up, compared to 8/48 (16.7%) and 4/32 (12,5%) of the patients with GGN=23 and GGN≥24, respectively. GGN>23 was a significant factor for survival in a Kaplan-Myer analysis (Fig 2). In a multivariate analysis including: age, Gleasonscore, M-status and PSA in serum before treatment, GGC<23 was a prognostic factor for CaP related death (Table 4).

For the SRD5A2 gene, there were no differences in baseline tumour characteristics, except that none with the AT genotype had metastasis at diagnosis. During follow-up, progression

was equal in all genotypes. There were no certain differences in the proportion of men that developed metastasis or died from prostate cancer, though the results are uncertain due to a limited number of some of the genotypes (Table 5).

Discussion

To our knowledge, the use of healthy men from the general population as controls is a new approach in investigating genotypes of importance for CaP.

In our study, the high-activity T allele in the A49T polymorphism in the SRD5A2 gene was over represented among men with CaP compared to men from the general population, suggesting that the progression to clinical cancer could be associated with induced DHT levels in the prostate. This assumption is supported by a recent meta-analysis, in which it could not be excluded that the T-allele in the A49T polymorphism may increase the risk for prostate cancer [20]. It was also proposed that men with at least one T-allele were more likely to have a younger age [21] and a trend for more advanced stage of the disease at diagnosis [22]. However, no such associations were seen in two other studies [23,24]. In our study, men belonging to this genetic subgroup did not present with more advanced disease. In fact, rather the opposite was found. None of the patients with the AT-allele had metastasis at diagnosis and only one case of death due to CaP was recorded after six years of follow-up. Notably, the expected numbers of deaths were 3. The number of subjects was, however, too small to draw any firm conclusions.

Individuals carrying the mutated A49T have also been reported to have larger prostates and higher PSA-levels than those with the wild-type gene [25]. A substantial reduction in the effect of the SRD5A2 inhibitor Finasteride in the codon 49 T variant has also been noted [26], suggesting that this drug was not blocking the enzymatic activity in men with this genotype. In our study, we did not find any associations with hormone concentrations or PSA in serum, except for significantly higher LH-level in patients with the AT-allele.

Interestingly, the homozygous V89L L-allele, known to have lower transcriptional activity *in vitro* [27], was less common in CaP patients compared to the general population. This gives further support that the genetically predisposed conversion of T to DHT plays a role in

progression of CaP to clinical disease. However, this predisposition did not correlate to a more aggressive disease in our CaP-patients, indicating that factors other than androgen metabolism are responsible for disease progression. The same trend was seen for BPH, though significance was not reached.

The R227Q polymorphism was completely absent in our population, suggesting that this polymorphism does not play any major role for the risk of CaP.

No differences between subjects with diagnosed CaP, men with BPH or men from the general population were found regarding mean-lengths of the polymorphisms in the AR gene, confirming a number of other studies on this topic [28-31]. However, in current study, short CAG-repeats, i.e. <23, were more often present in both the CaP and BPH groups, which was in accordance with a previous study by Hakimi *et al.* [17]. In opposite to the mentioned study, a short CAG-repeat was not a prognostic factor for poor prognosis in terms of death from CaP. Some studies have also reported short GGN alleles to be associated with CaP [17;32], whereas others reported negative findings [30,33]. In the present study, no differences in GGN median-length was found between the different groups studied. However, short GGN-repeats (<23) were associated with lower free testosterone serum levels and a higher risk of dying in CaP. This finding is in contrary to a previous report showing that GGN≥23 was associated with an increased risk for relapse and mortality [34]. In support of our study is the recently reported finding that a shorter GGN-repeat (19 GGN) *in vitro* had the same activity as longer variants, but yielded 2.7 times more AR protein than GGN=23 did [35]. The net result of AR action and hence total androgenicity was therefore calculated as higher in individuals with shorter GGN lengths.

Some reports have also stated that the CAG and GGN repeats are in linkage disequilibrium [29,36] in CaP cases, whereas other studies have found the opposite [17,32,37]. We did not find any particular CAG-GGN combinations in men with diagnosed CaP that were not present in the

conscripts. Hence, certain co-inherited combinations of alleles do not seem to contribute to an increased risk of CaP, at least not in our population.

Low circulating testosterone has also been put forward as a risk factor for CaP [38]. In our study, patients dying from CaP had lower free testosterone, compared to those who survived during follow–up. This could, however, be explained by higher age and a poor general condition at diagnosis. Free testosterone was not a prognostic factor for death in CaP in multivariate analysis including age at diagnosis either.

In this study healthy military conscripts represented the general population and it could be argued that some of these young men will develop CaP later in life and thus not constitute a proper group to compare CaP patients with. It should, however, be kept in mind that the genetic content in somatic cells does not change over time and the presence of men who will develop CaP in older age should diminish the difference between the two cohorts rather than produce false positive results.

In summary, based on our results, point mutations in the SRD5A2 gene, responsible for higher conversion of testosterone to DHT, seem to be more common in men with CaP than men from the general population. In our population, a short GGN-repeat in the AR gene was associated with a higher risk of cancer related death, why the possible use of this repeat as a prognostic marker in CaP progression should be confirmed in larger studies.

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Legends to figures

Figure 1. Free testosterone in 87 men with prostate cancer with different GGN-repeat lengths. Men with GGN>23 had higher free testosterone than those with GGN<23 (p=0.057) and GGN=23 (p=0.027).

Figure 2. Disease specific survival in prostate cancer patients. Kaplan-Myer, Log-Rank test, p=0.027. (n=84)[1]

Table 1. Patient characteristics. Gleasonscore was obtained from 88/89 patients with CaP. T-stage was given for 87/89 subjects and M-stage for 88/89 patients

	No of patients	Mean PSA (ng/ml) ± SEM
Gleasonscore 4-6	51	18 ± 2.3
7-8	36	150 ± 47
T-stage T1-2	61	22 ± 3
T3-4	26	197 ± 64
M0 (bone scan neg)	56	37 ± 8
Mx (bone scan n.d)	22	65 ± 44
M1 (bone scan pos)	10	300 ± 133
Benign	45	10.1 + 1.7

Table 2. Comparison of different genotypes in the SRD5A2 gene and mean serum levels of sex hormones in men with CaP.

Genotype	A49T		V89L		
	AA n=74	AT n=12	LL n=5	LV n=44	VV n=35
T (nM)	13.6	13.5	14.5	13.4	13.9
SHBG (nM)	42.3	46.2	39.0	42.4	45.4
LH (IU/L)	4.5	5.9*	3.0	4.5	5.1
T / SHBG	0.34	0.31	0.38	0.34	0.34

*p=0.019 in Mann-Withney U-test.

Table 3. Distribution of SRD5A2 genotypes. P values are in comparison with controls.

Genotype	CaP		BPH		Controls		CaP vs. controls	BPH vs. controls
	n	%	n	%	n	%	p value	p value
A49T								
AA	74	86	40	89	200	94		
AT	12	14	5	11	13	6	0.026	0,214
TT	0	0	0	0	0	0		
V89L								
VV/ VL	78	93	41	91	184	82		
LL	5	6	4	9	33	15	0.05	0,288
R227Q								
RR	87	100	45	100	108	100	1.000	1.000

Table 4. Multivariate analysis of risk of dying from CaP in 86 men with prostate cancer, including clinical parameters and AR GGN length. Three patients excluded; one due to missing PSA value and 2 because censored before the first event.

Parameter	**RR (95% conf interval)**	**p-value**
M1 at diagnosis vs. M0	12.1 (2.99-49.3)	<0.0001
PSA at diagnosis - continuous	1.003 (1.001-1.005)	0.0060
Age - continuous	1.129 (1.024-1.244)	0.0100
GGN<23 vs. GGN≥23	5.154 (1.171-22.7)	0.0120
Biopsy Gleasonsum numeric	2,185 (1.25-3.818)	0.0030

Table 5. Clinically important tumour parameters in relation to SRD5A2 polymorphisms. AT-genotype analysed in 86 patients.

		AA n=74	AT n=12	LL n=5	LV n=48	VV n=35
Mean age at diagnosis		69.2	71.1	69.6	68.9	69.7
Median PSA (ng/ml)		20.7	17.7	22.3	20.2	15.6
Gleasonscore n (%)	4-6	41 (55)	8 (67)	2 (40)	27 (56)	21 (60)
	7-10	33 (45)	4 (33)	3 (60)	21 (44)	14 (40)
Tumour stage T3-T4, n (%)		20 (28)	6 (50)	1 (20)	19 (28)	11 (31)
Surgery / RT		29 (39)	6 (50)	2 (40)	17 (35)	18 (51)
M1 at diagnosis, n (%)		10 (14)	0	0	6 (13)	4 (11)
Progression, n (%)		37 (50)	6 (50)	3 (60)	22 (50)	17 (48)
M+, n (%)		14 (19)	1 (8)	0	7 (32)	7 (20)
Death prostate cancer n (%)		15 (20)	1 (8)	0	9 (20)	7 (20)

Figure 1.

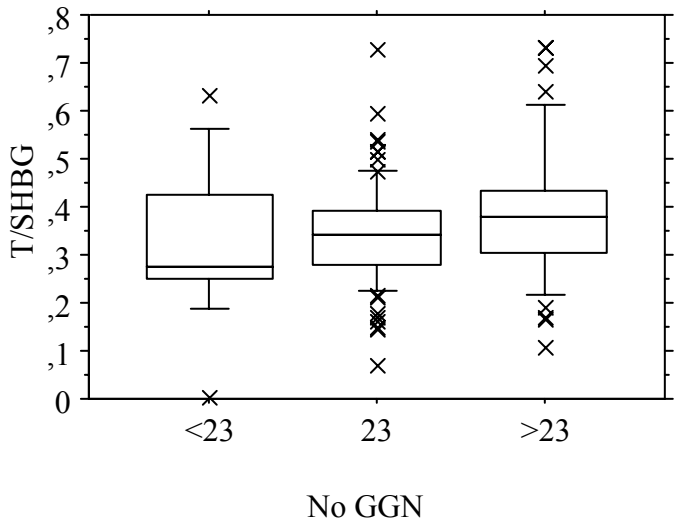
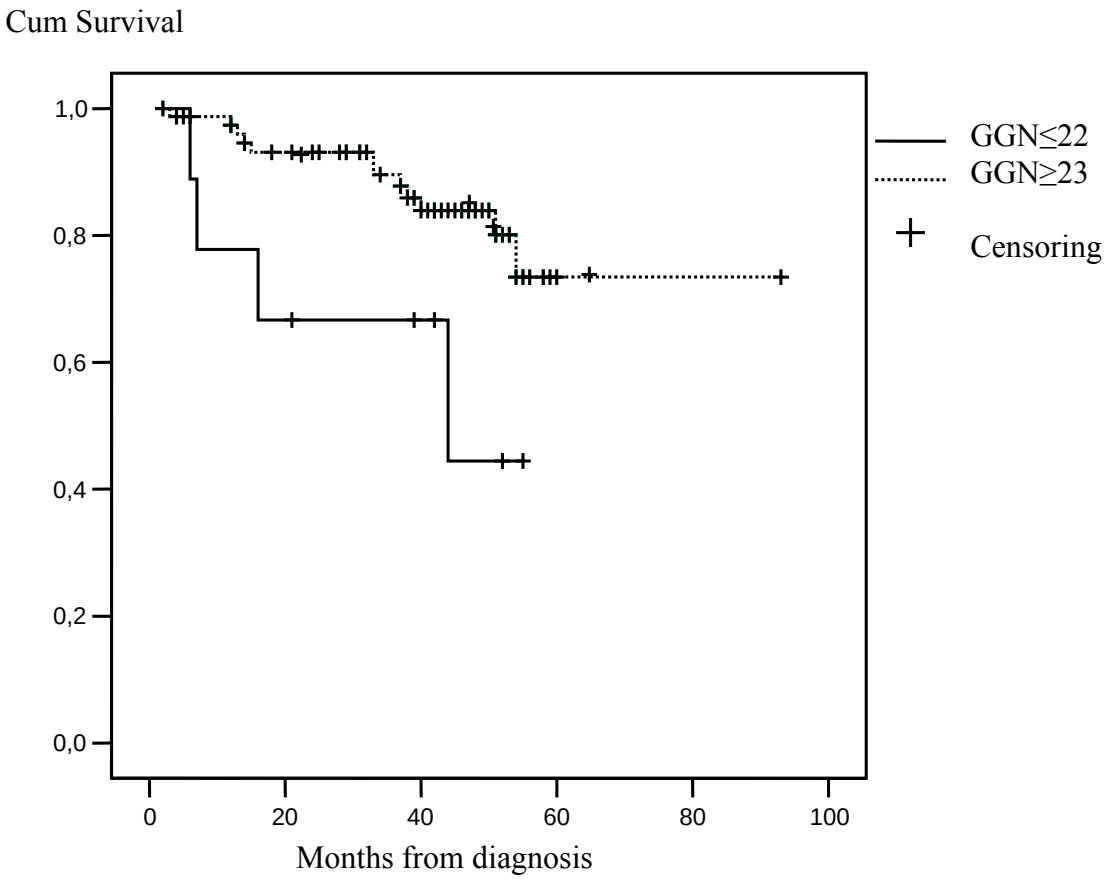


Figure 2.


Cum Survival
1,0
0,8
0,6
0,4
0,2
0,0
0
20
40
60
80
100
Months from diagnosis
GGN≤22
GGN≥23
Censoring