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The importance of a sub-region on chromosome 19q13.3 for prognosis of multiple myeloma patients after high-dose treatment and stem cells support: A linkage disequilibrium mapping in *RAI* and *CD3EAP*.

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Key words: multiple myeloma; SNP, chromosome 19q13.3, outcome, interferon- α , thalidomide, bortezomib.

Abbreviations: MM, multiple myeloma; OS, overall survival; TTF, time-to-treatment failure; HDT, high-dose treatment, SNP; single nucleotide polymorphism; LD, linkage disequilibrium (LD); CI, confidence interval; RR, relative risk; *ERCC1*, excision-repair cross-complementing 1; *ERCC2* excision-repair cross-complementing 2; *RAI*, RelA associated inhibitor (also named *PPP1R13L*, protein phosphatase 1, regulatory (inhibitor) subunit 13 like and

iASPP); *CD3EAP*, CD3ε molecule epsilon associated protein (also named *ASE-1*, anti-sense to ERCC1); NER, nucleotide excision repair;

Authors' contribution:

Annette J. Vangsted: Conception and design, collection of data, analysis and interpretation of data, drafting the article, approval of the version to be published.

Tobias W. Klausen: Conception and design, analysis and interpretation of data, approval of the version to be published.

Peter Gimsing: Collection of data, analysis and interpretation, revising the manuscript critically, approval of the version to be published

Niels Abildgaard: Analysis and interpretation, revising the manuscript critically, approval of the version to be published.

Niels F. Andersen: Analysis and interpretation, revising the manuscript critically, approval of the version to be published

Henrik Gregersen: Analysis and interpretation, revising the manuscript critically, approval of the version to be published

Bjørn A Nexø: Conception and design, determination of genotypes, analysis and interpretation of data, drafting the article, approval of the version to be published.

Ulla Vogel: Conception and design, analysis and interpretation of data, drafting the article, approval of the version to be published.

Abstract

The gene *RAI* was originally described as an inhibitor of RelA/p65 subunit of NF- κ B. Here, we analyze the association between genetic variation in the genes *RAI* and *CD3EAP* and outcome of 348 myeloma patients treated with high-dose treatment (HDT), 146 patients treated with INF- α as maintenance treatment, 177 patients treated with thalidomide and 74 patients treated with bortezomib at relapse and address if the effects of polymorphisms in *CD3EAP* and *RAI* are modified by a functional polymorphism in *NFKB1*.

By linkage disequilibrium mapping we found that variant alleles of several polymorphisms in a sub-region of 19q13.3, spanning the regions *RAI*-intron1-1 to *RAI* intron1-3 and the region exon1 to exon3-6 in *CD3EAP*, were associated with prolonged time-to-treatment failure (TTF) ($p=0.003$) and overall survival (OS) ($p=0.02$). Haplotype analyses revealed that none of the haplotype were stronger associated to TTF or OS than the two strongly linked SNPs; *RAI*-intron1-1 (rs4572514) and *CD3EAP G-21A* (rs967591).

The association of *RAI*-intron1-1 and *CD3EAP G-21A* with TTF was independent of *NFKB1* -94 ins/del, but homozygous ins-allele carriers who were also variant allele carriers of *RAI*-intron1-1 or *CD3EAP G-21A* had the longest OS. Among patients treated with INF- α , or thalidomide, no effect was seen in relation to genotype. Our results indicate that polymorphism in *RAI* and *CD3EAP* are associated with outcome of myeloma patients treated with HDT. Combination analyses with the functional polymorphism in *NFKB1* suggest that a possibly functional effect of *RAI* or *CD3EAP* could be related to NF- κ B availability.

Introduction

Multiple myeloma (MM) is an incurable B-cell malignancy. Younger patients are treated with high-dose treatment (HDT) and stem cells support. Despite this intensive treatment strategy, huge variation in treatment outcome is seen, with some patients suffering from early and aggressive recurrence of the disease. Understanding the mechanism of these differences may help us to establish new and better treatment strategies. Response to chemotherapy may reflect the effect of genetic variation related to differences in DNA repair, apoptosis and immune response.

A small region on the human chromosome 19q13.3 may be important for the development of cancers of the breast, lung and skin as well as outcome of chemotherapy (figure 1) [1-4]. The region encompasses 4 genes. Two genes, related to nucleotide excision repair (NER), the excision-repair cross-complementing 1 (*ERCC1*) and *ERCC2* (XDP), are involved in repair of bulky DNA damage caused by chemotherapy [5]. The gene product of the third gene *RAI* (RelA inhibitor; also named *iASPP* or protein phosphatase 1, regulatory (inhibitor) subunit 13 like (*PPP1R13L*)) [6;7] was identified as a protein that binds to the NF- κ B subunit p65/RelA and inhibits its transcriptional activity [8]. The inhibition of NF- κ B transcription induced apoptosis [8]. Later studies revealed that the *RAI* protein also inhibits apoptosis by binding to p53 [9]. Recently, over expression of *RAI* has been shown to increase tumor formation in primary mouse embryonic fibroblasts [7]. These data support the hypothesis that *RAI* may function as an oncogene. The fourth gene in the chromosome region 19q13.3 is named *CD3EAP* (CD3e molecule epsilon associated protein; alias *ASE1*(anti-sense to *ERCC1*); alias *CAST*)) [5]. The gene was cloned by use of autoimmune serum from a patient with interstitial cystitis [10]. It is orientated anti-sense to and overlaps with the *ERCC1* gene and it has therefore been suggested, that the mRNA of *CD3EAP* may bind the transcript of *ERCC1* and inhibit translation [11]. This hypothesis was not supported by scientific results. The protein is localized in the fibrillar centers of the nucleus, which may indicate a function in ribosomal RNA transcription [11]. Another study by Yamazaki et al. suggests that the gene product of *CD3EAP* mediates activation signals, important for IL-2 production [12]. In a recent study of fifty-nine NCI60 human tumour cell lines, sensitivity to anticancer drugs correlated positively with the variant A-allele of the polymorphism *CD3EAP* G-21A [13]. At present, the function of the gene is not clearly established. We have earlier shown that genetic variation in *CD3EAP* is associated with outcome in patients with multiple myeloma treated with HDT [4]. Patients carrying the variant A-allele of *CD3EAP* G-21A had a significant longer TTF and OS after HDT [4]. These results support the notion that the genomic region influence outcome of chemotherapy.

The gene *NFKB1* encodes the p50 subunit and its precursor p105. We have previously shown, that the polymorphism *NFKB1* -94 ins/del is associated with outcome of interferon- α (INF- α) maintenance treatment after HDT in patients with MM [14].

The aim of the present study was to gain further knowledge of impact of genetic variation in the human chromosome region 19q13.3, by fine mapping associations between many single nucleotide polymorphisms (SNPs) with outcome of 348 myeloma patients treated with HDT. Furthermore, we analysed if genes in the chromosome sub-region 19q13.3 interplay with the polymorphism in *NFKB1* in relation to outcome of multiple myeloma treated with HDT and maintenance treatment with interferon- α .

Materials and methods

Subjects, clinical data, response criteria, eligibility criteria, and treatment for patients included in SNP analysis.

Subjects, clinical data, response criteria, and eligibility criteria have previously been described in detail [4;14;15].

Briefly, patients diagnosed with MM and treated with HDT and stem cell support from August 1994 to August 2004 were recruited from four participating centres in Denmark. Three hundred forty-eight patients were included in the study. Staging was according to Durie and Salmon and the International Staging System (ISS). Time-to-treatment failure (TTF) and overall survival (OS) were calculated from date of stem cell infusion to date of progression or death. Progression was defined by a more than 25% increase in serum M-protein or 25% increase in immunoglobulin levels above upper normal levels, confirmed by 2 separate measurements within a 1-month interval. Increase in bone marrow infiltration of plasma cells by 25%, and increase of Bence-Jones proteinuria to more than 1.0g/24 hr or other signs of progression such as hypercalcaemia, progressive skeletal disease or soft-tissue plasmacytoma were also considered as progression. The occurrence of other malignancies and death without progression was regarded as events not related to progression. These patients were included in the analysis of OS, but they were excluded at time of death in the analysis of TTF. Complete response (CR) after HDT was defined by the disappearance of M-protein from serum and urine, as detected by agarose gel electrophoresis and less than 5% plasma cells in the bone marrow aspirate. Partial response (PR) was defined by a reduction of at least 50% in the initial serum M-protein concentration and a reduction of light chain proteinuria to less than 0.2g/24 hours. Time-to-next treatment (TNT) was used as follow-up after relapse treatment and was defined as the period from start of relapse treatment to start of new relapse treatment, progression without new treatment or death.

Induction therapy was 3 series VAD (vincristine, doxorubicin and dexamethasone) or 2-3 series of cyclophosphamide 1g/m² i.v. on day 1 combined with dexamethasone 40 mg daily p.o., on days 1-4 and days 9-11 (total dose 320 mg for each series) repeated every 3 weeks. Peripheral blood stem cells were harvested at regeneration after cyclophosphamide priming, and the patients thereafter underwent high-dose chemotherapy with melphalan (200 mg/m²) followed by stem cell support. Fifty-three patients received a second HDT up-front. Maintenance therapy with IFN- α was implemented 3 months after HDT according to standard treatment practise in the particular region (median 105 days) [14]. Two-hundred and forty-three patients experienced relapse which required treatment. Among these patients 177 were treated with thalidomide and 74 were treated with bortezomib [16].

PBMC was purified from 292 leukapheresis products by buffy coat preparation. From 56 patients 10 times 10 μ m sections were collected from paraffin embedded bone marrow samples. Material was not available for 19 patients undergoing HDT and these patients were not included in the study.

The study was approved by the Danish Ethical Committee (01-158/03).

DNA purification

DNA for analysis was purified from PBMC by the salting out method [17] or from paraffin embedded tissue by phenol extraction as described elsewhere [18].

Genotyping of single nucleotide polymorphisms

The *XPD (ERCC2)* SNP rs238406, rs238407, rs2097215, the *RAI* SNPs rs10422489, rs4544343, rs6966, rs12986272, rs10402584, rs10402393, rs4572514, rs4803816, rs95947, rs928911, rs4803817, nr.106, rs10412761 and the *CD3EAP (ASE1)* SNPs rs8113779, rs2013521, rs735482, rs762562, rs2336219, rs3212986, rs3212985, rs1007616, and the *ERCC1* SNPs rs3212969, rs3212961, rs2298881, and rs1319052 were tested using a Sequenom® platform (San Diego, California) and the iPLEX gold reaction. Each PCR reaction contained 10 ng of genomic DNA, 0.5 U HotStar Taq from Qiagen (Hilden, Germany), 1.25 x Enzyme Buffer (Qiagen), 3.5 mM MgCl₂, and 1 mM of each deoxynucleotide. The primers were added to a final concentration of 500 nM each. The total reaction volume was 4 μ l. PCR reactions with primers of each plex were performed in 384 well plates with V-shape by performing a 15 min preheat to 94 C followed by cycling the samples 45 times, 94 C for 20 sec, 56 C for 30 sec and 72 C for 1 min, followed by 3 min at 72 C. The plates were stored at -20 C. Treatment with Shrimp Alkaline Phosphatase and extension with molecular weight-modified nucleotides was performed according to the manufacturer's recommendations. The reactions were cleaned

with resin and spotted on SpectroChip arrays according to the manufacturer's recommendations. The reactions were analyzed by MALDITOF mass spectrometry on the Sequenom equipment, and the results were analyzed using the MassARRAY Type 4.0 (Sequenom). The primers and probes are presented in supplement Table 1. For *XPD*-exon23 (rs13181); *XPD*-exon10 (rs1799793), *RAI*-intron8-1 (rs1970764), *ASE1*-exon1 (rs967591), *ERCC1*-exon4 (rs317770), and *NFKB1* ATG (rs28362491) the method has been previously reported [4;14;19]

Statistical methods

SPSS statistical software was used for all calculations (SPSS for Windows, Rel. 14.0.0. 2005, Chicago: SPSS Inc.). All tests were two-sided and P-values < 0.05 were regarded as statistically significant. Fisher's exact test was used to compare categorical variables and the Mann-Whitney test was used to compare continuous and categorical variables. Kaplan-Meier method and the log rank test were used to compare TTF and OS between groups. The Cox proportional hazard model and log-likelihood statistics were applied for univariate analyses and covariates and for multivariate analysis.

Results

Study population

Three-hundred and forty-eight myeloma patients were treated with HDT and stem cell support. The patient characteristics are presented in Table 1. The median age of the patients was 56 years and the study included 59% males. The median follow-up of all patients still alive was 93.4 months (range, 54.6-174.2 months). At follow up 208 patients had died. The median OS was 69.8 months (60.5-81.6 months). The median TTF was 27.7 months (23.4-30.8 months). One-hundred and forty-six patients were treated with INF- α maintenance treatment.

The genotype frequencies of *XPD*, *RAI*, *CD3EAP* and *ERCC1* and their influence on TTF, OS and response to treatment

All genetic variation in the chromosomal region was previously identified by sequencing of breast cancer patients and healthy controls [20]. The genotype frequencies and association of minor allele frequency with TTF and OS after HDT are summarized in supplementary Table 2. For completeness of the investigation, we have also included previously reported SNPs in the region [4]. The percentage of successful genotype determinations was low for a number of polymorphisms, reflecting that the mass-spectrometry-based genotyping was unsuccessful for DNA isolated from

paraffin embedded tissues, which constituted 16% of the samples. The outcome for homozygous wild type carriers versus variant carriers association of single polymorphisms in *XPD*, *RAI*, *CD3EAP* and *ERCCI* is illustrated in Figure 2. A sub-region of 19q13.3 on the chromosome spanning the regions *RAI*-intron1-1 to *RAI*-intron1-3 and the region exon1 to exon3-6 in *CD3EAP*, was significantly associated with TTF and OS in myeloma patients treated with HDT, and carriers of the variant allele of the SNPs had prolonged TTF and OS. There was no difference in the allele frequencies among patients dependent on the participating centres, and there was no difference in genotype distribution in relation to age, β 2-microglobulin, creatinine, albumin, Durie-Salmon stage, ISS stage, gender, tandem HDT upfront, INF- α maintenance treatment, second HDT at relapse, treatment with thalidomide or bortezomib (supplementary table 3). There was no association between allele frequencies and response to treatment.

Haplotype analysis of *XPD*, *RAI*, *CD3EAP* and *ERCCI* and the influence on TTF and OS in myeloma patients treated with HDT.

Pair-wise linkage disequilibrium and block structure of 33 SNP' in the genes *XPD*, *RAI*, *CD3EAP* and *ERCCI*, using the haplo-view software program, are illustrated in supplementary Figure 1. Two haplotype analyses were generated for the sub-region 19q13.3. One haplotype analysis covered the genes *RAI*, *CD3EAP* and *ERCCI* (rs4803816, rs967591, rs3212985 and rs1319052), and the other haplotype covered the gene *CD3EAP* (rs8113779, rs735482, rs3212986 and rs1007616). The two haplotype analyses included 97.6% and 85% of all patients with available genotype data in the study, respectively. None of the haplotype analyses revealed stronger association to TTF or OS than the two most significant SNP's; *RAI*-intron1-1 (rs4572514) and *ASE1*-exon1 (rs967591).

Combination of SNPs

Supplementary Table 4 and Figure 3 show the combination analysis of *NFKB1* -94ins/del, *CD3EAP* G-21A and *RAI*-intron1-1. When genotypes of *CD3EAP* or *RAI* were combined with genotypes of *NFKB1* -94ins/del, carriers of the variant A-allele of *CD3EAP* G-21A or the variant C-allele of *RAI*-intron1-1, a prolonged TTF was seen independently of the *NFKB1*-94ins/del genotype, in contrast to OS, where an increase in median survival to more than 10 years, only was seen among carriers of the variants alleles of either *CD3EAP* G-21A or *RAI*-intron1-1, when combined with the wild type ins-allele of *NFKB1* -94ins/del.

Analysis of the effect of genotypes on TTF and OS in patients treated with INF- α , thalidomide and bortezomib.

TTF and OS were compared in groups subdivided by genotype and INF- α treatment. Among patients treated with INF- α , carriers of the variant C-allele or *RAI*-intron1-1 had a significant prolonged TTF and OS (56.3 months and not reached, respectively) as compared to homozygous T-allele carriers (30.5 months and 74.4 months, respectively). However, when tested in a multivariate Cox analysis, the analysis showed that the difference in TTF was not related to treatment with INF- α and only a trend was found for OS ($p=0.06$). No association between treatment outcome of thalidomide and bortezomib with genotype was found, except for carriers of the variant A-allele of *CD3EAP* -G-21A, where a significantly prolonged TTF and OS was seen. We explored combination analyses of *NFKB1* -94ins/del, *CD3EAP* G-21A and *RAI*-intron1-1 in relation to treatment with INF- α , and the results are shown in Table 2. Patients treated with INF- α , who carried two copies of the T-allele of *RAI*-intron1-1 and the variant del-allele of *NFKB1* -94ins/del had a significantly shorter TTF, and patients who carried the variant del-allele of *NFKB1* -94ins/del and either of the wild type alleles of *CD3EAP* G-21A or *RAI*-intron1-1 had a significant shorter OS survival as compared to carriers of the wild type ins-allele of *NFKB1* -94ins/del in combination with the variant alleles of either *CD3EAP* G-21A or *RAI*-intron1-1 (figure 4).

Discussion

We have explored a sub-region in 19q13.3 by fine mapping associations between many single nucleotide polymorphisms (SNPs) and treatment outcome in 348 myeloma patients treated with HDT. We found that several polymorphisms in a region spanning the genes *RAI*-intron1-1 and *CD3EAP*-exon3-6 were significantly associated with TTF and OS in myeloma patients. The effects of the polymorphisms *RAI*-intron1-1 and *CD3EAP* G-21A on gene functions are not known. Both SNPs are located in the 5' region of their respective genes, the first intron and the 5'UTR, respectively, but none of them are located in the reading frame, and one may speculate that they affect transcription efficiency or gene regulation. In the studied population of patients, the effects of polymorphisms in *RAI* and *CD3EAP* were similar, and we found a pair wise linkage disequilibrium between *RAI*-intron1-1 (rs4572514) and *CD3EAP* G-21A (rs967591) of $D' = 0.94$, suggesting that they detect the same biological effect.

The gene *RAI* was initially identified as an inhibitor of the NF- κ B subunit p65. The pro-inflammatory NF- κ B consists of the dimer p50/p65. The *NFKB1* -94ins/del ATTG polymorphism is an insertion/deletion of four bases in the promoter region of the *NFKB1* gene encoding both of the NF- κ B transcription factors, p50 and p105. We therefore combined the polymorphism *NFKB1* -94ins/del with polymorphism in *RAI*-intron1-1 in relation to outcome of

treatment. From previous results we know that TTF and OS are not significantly affected by the polymorphism *NFKB1* -94ins/del [19]. The deletion produces lower transcripts levels in luciferase reporter systems, and thus results in lower levels of NF-κB p50 [21]. In myeloma patients treated with HDT, the effect on TTF for the polymorphism *RAI*-intron1-1 was independent of the polymorphism *NFKB1* -94ins/del. Assuming that *RAI* is indeed an inhibitor of NF-κB p65, this would indicate that NF-κB p65 levels influence TTF, and that this effect is independent from differences in NF-κB p50 levels. For OS, the effect of *RAI*-intron1-1 was in favour of patients also carrying the wild type ins-allele of *NFKB1* -94ins/del, and these findings support that the classical NF-κB pathway (p65/p50 dimer) is involved in survival of myeloma patients after HDT.

In this population of patients a subgroup of 146 patients were treated with INF-α as maintenance treatment after HDT. The decision of starting INF-α treatment was solely based on different treatment guidelines on starting post-HDT maintenance treatment at the participating departments and therefore not connected with selection bias. Interferon and NF-κB are central for the regulation of apoptosis, cell growth and for modulation of the immune system [22;23]. From previous results, we know that the polymorphism *NFKB1* -94ins/del influences outcome for patients treated with INF-α as maintenance treatment [14]. Among patients treated with INF-α, carriers of the variant del-allele carriers of *NFKB1* -94ins/del and the wild type T-allele of *RAI*-intron1-1 had a significant shorter TTF and OS survival as compared to double homozygous wild type carriers. The effect of INF-α may therefore depend on a normal transcription level of the NF-κB p50 subunit, and the availability of the p65 subunit of NF-κB for transcription activity. In the combination analyses *CD3EAP* G-21A AG+GG was similar to *RAI*-intron1-1 TC+CC, probably due to their strong linkage (supplementary figure 1). In general, *RAI*-intron1-1 had the stronger effect, perhaps indicating that *RAI*-intron1-1 is the biologically relevant polymorphism. In carriers of the variant A-allele of *CD3EAP* -G-21A, a significantly prolonged OS was seen for patients treated with bortezomib. If true, this result could be interesting because Hideshima et al recently found that bortezomib treatment increases NF-κB dependent transcription in myeloma cells in vitro via activation of two upstream NF-κB-activating kinases RIP2 and IKKβ [24]. However, in our cohort of patients only few patients were treated with bortezomib, and consequently the statistical power to detect interaction with bortezomib was limited. No effect on TNT was seen, and combination analysis of *RAI*-intron1-1 did not reveal the same statistically significant results (table 2). We conclude that the polymorphisms *RAI*-intron1-1 and *CD3EAP*-exon3-6 are associated with outcome of myeloma patients treated with HDT. The effects of the polymorphisms on gene function are unknown, as well as the function of the genes *RAI* and in particular *CD3EAP*. The findings should be sought replicated in an independent study group of similarly treated multiple myeloma patients. Combination analyses with the functional

polymorphism in *NFKB1* suggest that a possibly functional effect of *RAI* or *CD3EAP* could be related to NF- κ B availability. This hypothesis should be tested at the molecular level.

Legends to figures

Figure1.

Schematic presentation of the sub-region 19q13.3 investigated in the current study.

Figure2.

Association of single polymorphisms with TTF (A) and OS (B) in multiple myeloma patients treated with high-dose treatment and stem cell support. The p value (logarithmic scale) for association of homozygous wild type carriers compared to all variant carriers of each marker with TTF and OS, are depicted against the position of the marker.

Figure 3.

Kaplan-Meier plots of TTF and OS. Combination analysis of *CD3EAP* G-21A or *RAI*-intron1-1 with *NFKB1* -94ins/del. The numbers at risk at 24, 48, 72, 96 and 120 months are presented below the figure. Wild type carriers of either *CD3EAP* G-21A or *RAI*-intron1-1 in combination with wild type carriers of *NFKB1* -94ins/del (solid blue line), variant type carriers of either *CD3EAP* G-21A or *RAI*-intron1-1 in combination with wild type carriers of *NFKB1* -94ins/del (dashed blue line), wild type carriers of either *CD3EAP* G-21A or *RAI*-intron1-1 in combination with variant type carriers of *NFKB1* -94ins/del (solid red line), variant type carriers of either *CD3EAP* G-21A or *RAI*-intron1-1 in combination with variant type carriers of *NFKB1* -94ins/del (dashed red line).

Figure 4.

Kaplan-Meier plots of A: TTF and B: OS. Patients treated with interferon- α in relation to genotypes of *CD3EAP* G-21A or *RAI*-intron1-1 in combination with genotypes of *NFKB1* -94ins/del. The numbers of patients at risk at 0, 24, 48, 72, 96 and 120 months are presented below the figure. Wild type carriers of either *CD3EAP* G-21A or *RAI*-intron1-1 in combination with wild type ins-allele carriers of *NFKB1* -94ins/del (solid blue line), variant type carriers of either *CD3EAP* G-21A or *RAI*-intron1-1 in combination with wild type ins-allele carriers of *NFKB1* -94ins/del (dashed blue line), wild type carriers of either *CD3EAP* G-21A or *RAI*-intron1-1 in combination with variant-type del-allele carriers

of *NFKB1* -94ins/del (solid red line), variant type carriers of either *CD3EAP* G-21A or *RAI*-intron1-1 in combination with variant type del-allele carriers *NFKB1* -94ins/del (dashed red line).

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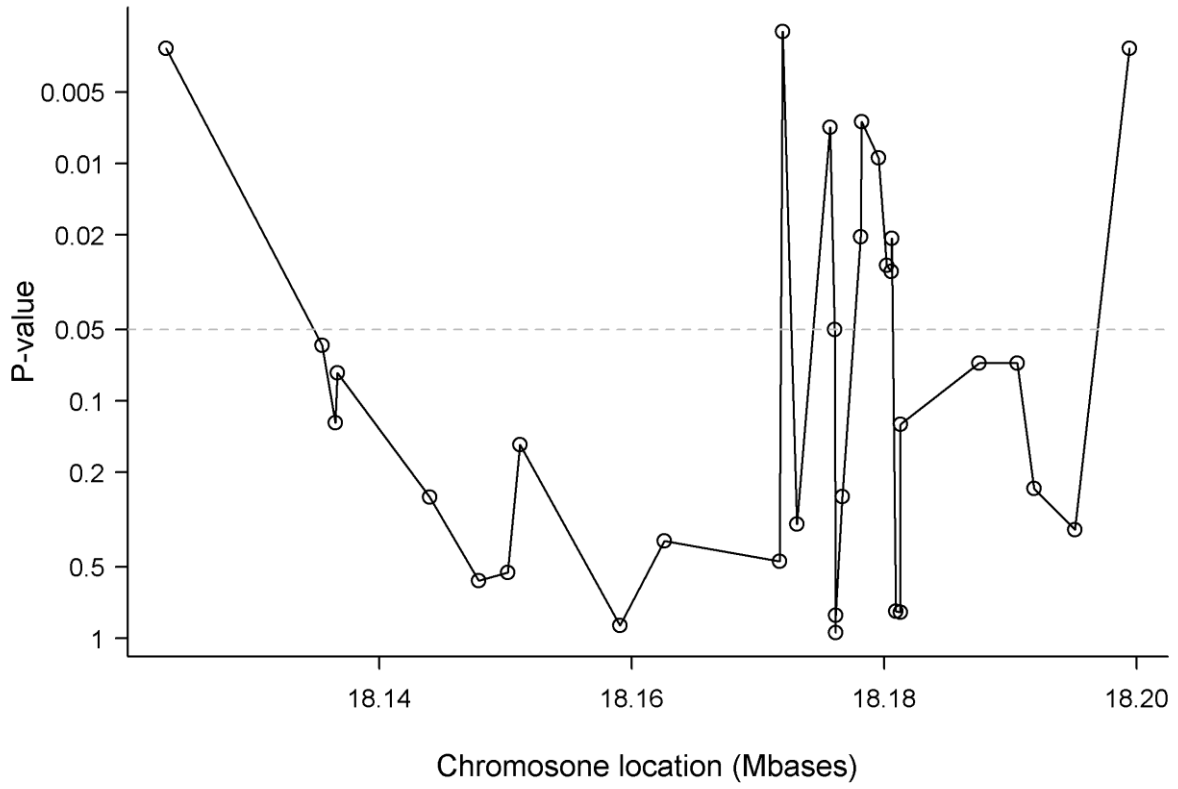
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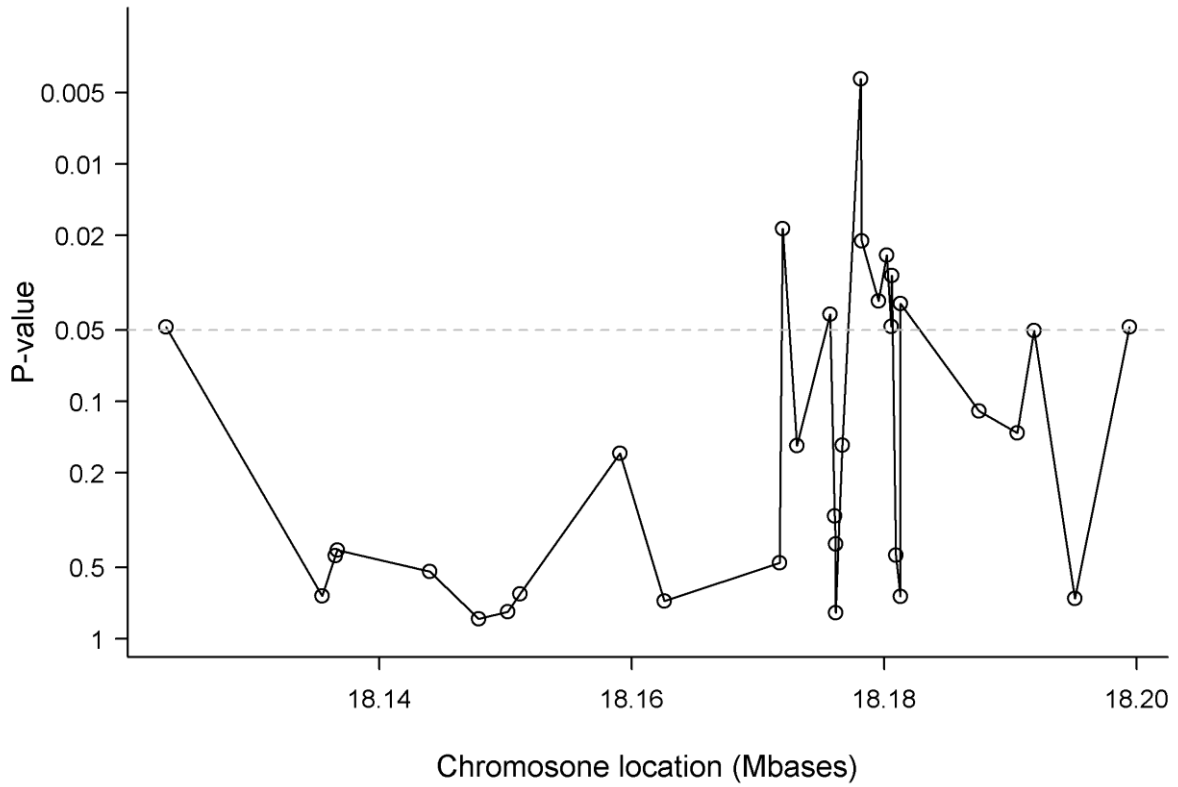
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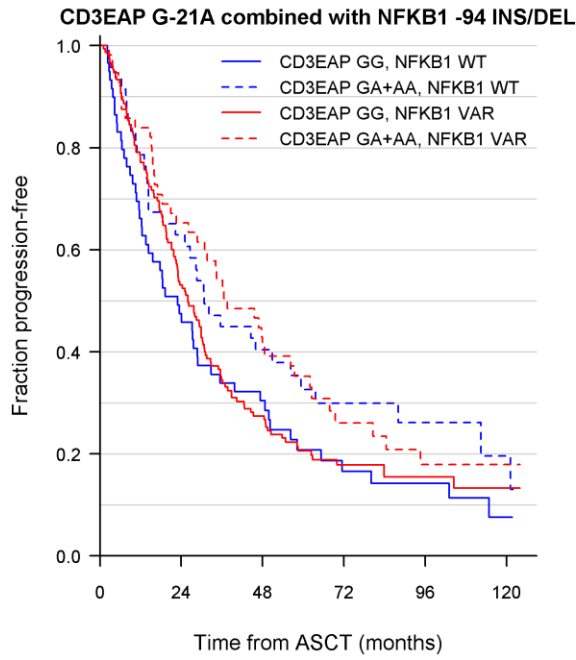
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Figure 2.
A



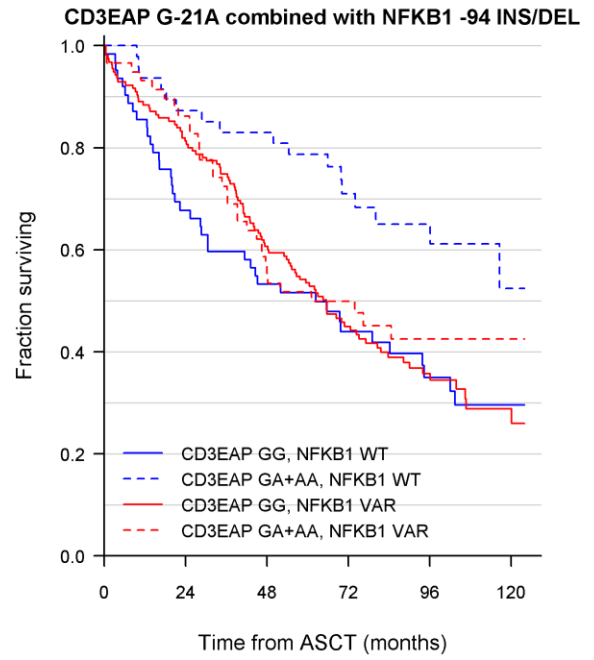
B





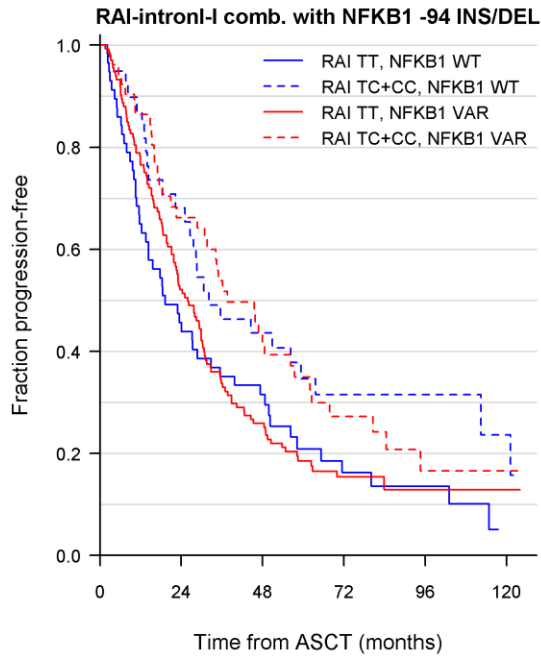
No. at risk

GG, WT	60	28	16	7	5	1
GA+AA, WT	47	28	18	10	6	3
GG, VAR	155	77	38	17	9	4
GA+AA, VAR	58	35	23	11	6	4



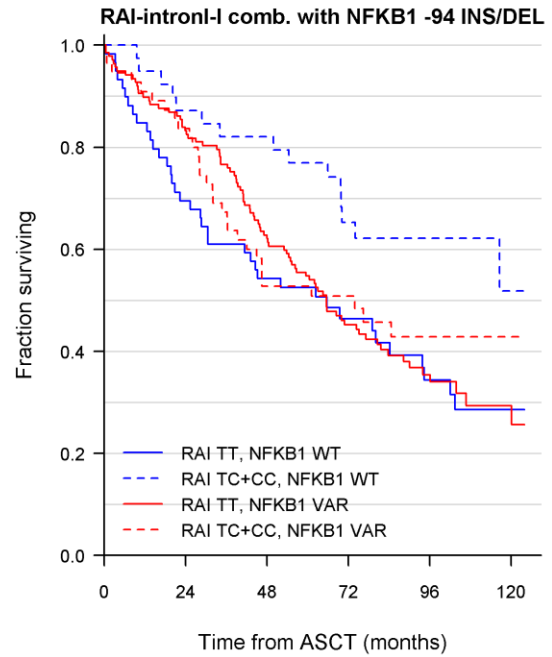
No. at risk

GG, WT	62	42	33	22	15	6
GA+AA, WT	47	41	39	26	17	6
GG, VAR	155	126	94	55	30	10
GA+AA, VAR	58	50	33	24	12	9



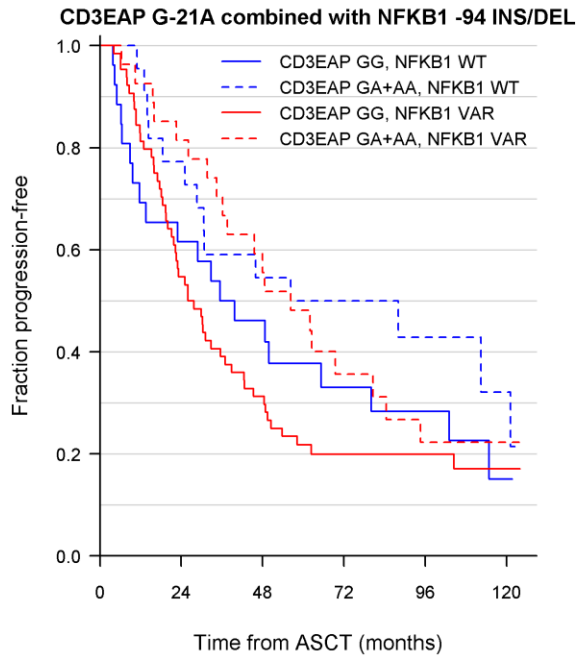
No. at risk

TT, WT	58	26	16	6	4	0
TC+CC, WT	39	25	16	9	6	3
TT, VAR	137	68	33	13	6	4
TC+CC, VAR	55	32	21	10	4	2



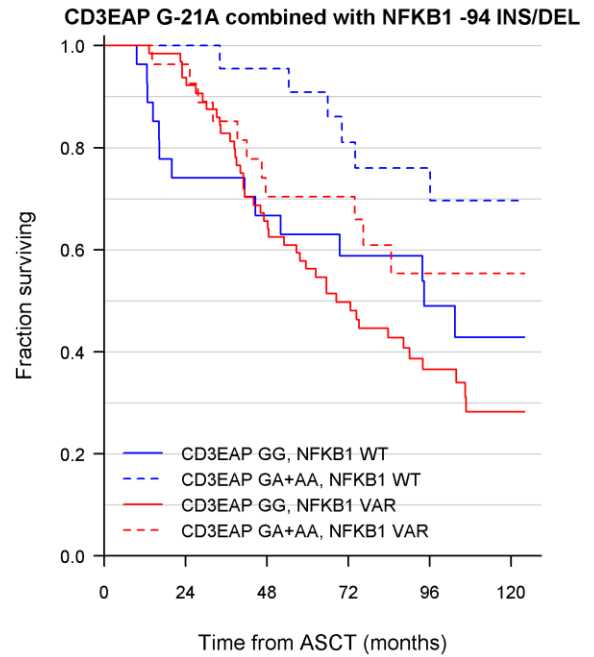
No. at risk

TT, WT	59	41	32	21	14	5
TC+CC, WT	39	34	32	21	14	5
TT, VAR	137	114	86	49	26	8
TC+CC, VAR	55	46	29	23	10	7



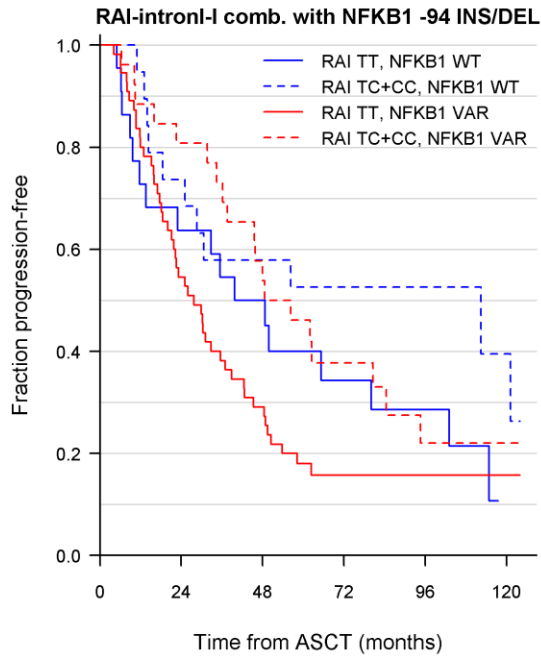
No. at risk

GG, WT	26	16	11	7	5	1
GA+AA, WT	22	17	12	9	5	3
GG, VAR	64	35	20	11	8	4
GA+AA, VAR	27	22	16	8	5	3



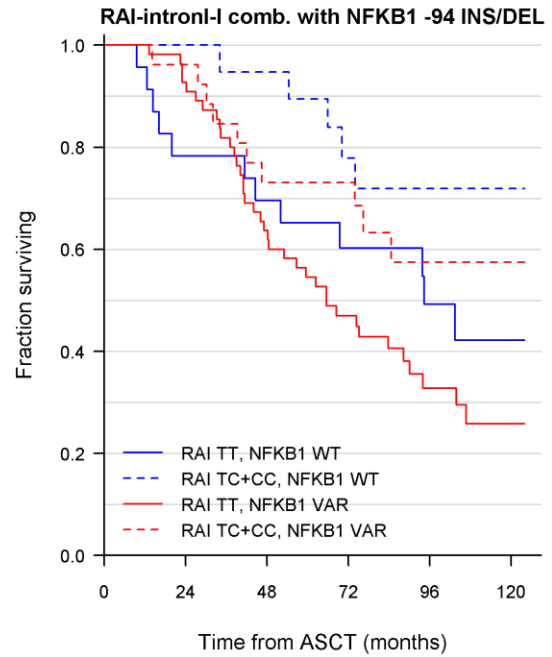
No. at risk

GG, WT	27	20	18	14	10	6
GA+AA, WT	22	22	21	16	12	6
GG, VAR	64	60	42	29	16	7
GA+AA, VAR	27	26	19	18	9	7



No. at risk

TT, WT	22	14	10	6	4	0
TC+CC, WT	19	14	11	8	5	3
TT, VAR	55	30	16	7	5	4
TC+CC, VAR	26	21	15	8	4	2



No. at risk

TT, WT	23	18	16	12	9	5
TC+CC, WT	19	19	18	13	9	5
TT, VAR	55	51	35	23	12	5
TC+CC, VAR	26	25	19	18	8	6

Table 1. Patient characteristics at the time of diagnosis of 348 patients.

	No of patients	Median	Range
Sex			
Male	204 (59%)		
Female	144 (41%)		
Age		56	(28-69)
<60	246 (71%)		
>= 60	101 (29%)		
β 2-microglobulin (mg/dL)	249	3.9	(1.2 – 57)
Creatinine (μ mol/L)	332	98	(47 – 833)
Albumin (g/dL)	297	3.5	(0.25 – 5.3)
ISS			
I	56 (23%)		
II	94 (39%)		
III	89 (37%)		
Durie-Salmon stage			
I	35 (10%)		
II	77 (23%)		
III	228 (67%)		
TTF (months)		27.7	(23.4-30.8)
OS (months)		69.8	(60.5-81.6)
Treated with thalidomide at relapse	177		
Treatment with bortezomib at relapse	74		

Gene	Genotypes		N	Median TTF or TNT (mo)	HR (95%CI)	p value	N o
<i>RAI-intron1-1</i>							
+ INF- α		TT	78	30.5	1		
+ INF- α		TC + CC	46	56.3	0.6 (0.4-0.9)	0.01	
		TT	116	21.7	1		
		TC + CC	42	28.7	0.8 (0.5-1.2)	0.23	
<i>RAI-intron1-1</i>							
+ Thalidomide		TT	113	10.7	1		
+ Thalidomide		TC + CC	41	7.2	1.0 (0.6-1.4)	0.87	
<i>RAI-intron1-1</i>							
+ Bortezomib		TT	43	8.9	1		
+ Bortezomib		TC + CC	16	13.2	0.6 (0.3-1.3)	0.22	
<i>CE3EAP G-21A</i>							
+ Thalidomide		GG	121	10.2	1		
+ Thalidomide		AG + AA	51	9.1	0.9 (0.6-1.2)	0.39	
<i>CE3EAP G-21A</i>							
+ Bortezomib		GG	49	8.6	1		

+ Bortezomib		AG + AA	22	13.3	0.6 (0.3-1.1)	0.06	
<i>NFKB1-94</i> INS/DEL combined with <i>CD3EAP</i> G-21A							
+ IFN- α	ins	GG	27	37.6	1		
+ IFN- α	ins	AG + AA	22	56.4	0.6 (0.3-1.3)		
+ IFN- α	Ins/del + del	GG	64	26.0	1.2 (0.7-2.0)		
+ IFN- α	Ins/del + del	AG + AA	27	56.3	0.7 (0.4-1.4)	0.1	
<i>NFKB1-94</i> INS/DEL combined with <i>CE3EAP</i> G-21A							
	ins	GG	33	17.7	1		
	ins	AG + AA	23	22.3	0.7 (0.4-1.2)		
	Ins/del + del	GG	86	26.2	0.6 (0.4-0.9)		
	Ins/del + del	AG + AA	27	28.7	0.6 (0.3-1.0)	0.1	
<i>NFKB1-94</i> INS/DEL combined with <i>RAI</i>-intron1-1							
+ IFN- α	ins	TT	23	39.8	1		
+ IFN- α	ins	TC + CC	19	112.4	0.6 (0.3-1.3)		
+ IFN- α	Ins/del + del	TT	56	27.8	1.4 (0.8-2.4)		
+ IFN- α	Ins/del + del	TC + CC	26	52.5	0.8 (0.4-1.5)	0.04	
<i>NFKB1-94</i> INS/DEL combined with <i>RAI</i>-intron1-1							
	ins	TT	34	15.6	1		
	ins	TC + CC	18	32.2	0.6 (0.3-1.1)		
	Ins/del + del	TT	79	24.3	0.6 (0.4-1.0)		
	Ins/del + del	TC + CC	24	28.7	0.6 (0.3-1.0)	0.12	

Table 2 Univariate analysis of the effect of genotypes on treatment outcome of INF- α , thalidomide and bortezomib.

Abbreviations: CI= confidence interval; HR= hazard ratio; TTF= time-to-treatment failure; TNT: time-to-next treatment; OS= overall survival; mo = months;
 ∞ : indicates that median survival was not reached