

Genomic landscape alterations in primary tumor and matched lymph node metastasis in prostate cancer patients

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Abstract

Background: Prostate cancer (PCa) is a disease with a wide range of clinical manifestations. Up to date, the genetic understanding of patients with favorable or unfavorable prognosis is gaining interest in order to give the appropriate tailored treatment. The aim of the present study was to investigate genetic changes associated with lymph node metastasis in a cohort of hormone-naïve Pca patients.

Methods: We retrospectively analyzed data from 470 patients who underwent surgery for PCa between 2010 and 2020 at the Department of Urology, University of Catania. The final cohort consisted of 17 different patients (11 PCa with lymph node metastasis and 6 PCa without lymph node metastasis). Through the cBioPortal online tool, we analyzed gene alterations and their correlations with clinical factors.

Results: A total of 688 Intronic, Synonym and Non-Synonym mutations were sequenced. The gene with the most sequenced mutations was ERBB4 (83 mutations, 12% of 688 total) while the ones with the lower percentage of mutations were AKT1, FGFR2 and MLH1 (1 mutation alone, 0.14% of 688 total). We used the cBioPortal online tool to study the alterations of the genes ERBB4, HRAS, KIT, ABL1, CTNNB1 and their correlation with prognosis using a total of 23 studies (9377 samples). When incorporating KIT, HRAS and CTNNB1 alterations in combination score we found that overall survival was statistically worse ($p < 0.01$) and also disease-free survival ($p = 0.02$) compared to unaltered group.

Conclusion: In the present study we found mostly concordance between both primary PCa samples and matched lymph node metastasis in terms of genomic alterations, underlining that alterations in primary tumor are extremely important for cancer prognosis prediction. We demonstrated worse overall survival and disease-free survival in patients with combined alteration of KIT, HRAS and CTNNB1. These results can be applied for monitoring disease and drug response after curative treatment.

Introduction

Prostate cancer (PCa) is a disease with a wide range of clinical manifestations. Every year, over 900,000 new cases of prostate cancer are diagnosed worldwide ¹. Among risk factors, there are those associated with life style, i.e. diet, tobacco smoking, obesity that increase the development of several neoplasms such as PCa ²⁻⁴.

PCa can be kept under control in some cases, but in others it can progress into an aggressive form, generating metastasis and even leading to the patient's death; PCa is still the second leading cause of cancer death worldwide. At diagnosis time, the majority of PCa cases are either localized within the prostate gland or in the locoregional area, with invasion into surrounding structures or lymph nodes ⁵.

Lymph nodes metastasis have been associated with adverse prognosis and worse outcome ⁶. However, some clinical data have reported that about 30% of patients with lymph node metastasis are recurrent free after 10-year of follow-up suggesting the importance of a better understanding of its heterogeneity ⁷.

In fact, a large percentage of PCa diagnoses may have a strong genetic component. Men with several single-gene polymorphisms and a family history of PCa have an increased risk of developing the disease. PCa risk has been linked to several single-gene mutations. These include BRCA1 and BRCA2 (Breast Cancer 1 and 2), ATM (Ataxia Telangiectasia Mutated) or HOXB13 (Homeobox B13)^{8,9}.

Interestingly, Kneppers et al compared the copy-number alterations of primary PCa and pelvic lymph node metastases showing that in 23.3% the index primary tumor was not clonally related to the locoregional lymph node metastases¹⁰.

Up to date, the genetic understanding of patients with favorable or unfavorable disease is gaining interest in order to give the appropriate tailored treatment.

Based on these premises, the aim of the present study was to investigate genetic changes associated with lymph node metastasis in a cohort of hormone-naïve PCa patients.

Materials And Methods

We retrospectively analyzed data from 470 patients who underwent surgery for PCa between 2010 and 2020 at the Department of Urology, University of Catania. This study was conducted in accordance with the Declaration of Helsinki and the protocol was approved by the Ethics Committee of the Policlinic Hospital of Catania (#144/2021). The final cohort consisted of 17 different patients (11 PCa with lymph node metastasis and 6 PCa without negative lymph node metastasis).

For each patient, an expert pathologist marked on hematoxylin and eosin-stained sections primary PCa tissue, a marked areas of distant adjacent normal tissue and lymph node metastasis tissue (in positive patients).

Furthermore, primary tumor samples were used for the assembly of the tissue microarray using the Galileo TMA CK3500 (Integrated System Engineering, Milan, Italy), as previously published^{11,12}. Immunohistochemical analyses with anti-AR (ab74272; rabbit polyclonal), anti-IR- α (ab5500; rabbit polyclonal), anti-IR- β (ab69508; mouse monoclonal clone: C18C4), anti-IGF1-R (ab39398; rabbit polyclonal) and anti-PSMA (ab64082; rabbit monoclonal; clone: SP29) antibodies (all from Abcam, Cambridge, UK) were performed as previously described^{13,14}; the scoring system included a combined analysis of staining intensity (IS) and percentage of immunoreactive cells (extent score; ES), as previously described^{13,14}.

Deparaffinization Procedure and DNA Extraction

The deparaffinization was carried out following the manufacturer's practice provided by QIAGEN QIAamp® DNA FFPE Advanced Kit (Ref. 56604, QIAGEN, 40724 Hilden, Germany). The results of deparaffinization and DNA quantification are shown in Supplementary Table 1.

13 samples reported insufficient coverage in the first sequencing to conduct a satisfactory analysis, so they were subjected to a quality control. This was carried out using the Illumina® FFPE QC Kit (Ref. 15013664, Illumina, Inc. San Diego, CA 92122 USA) and following the manufacturer's instructions. The results are reported in Supplementary file 2. Even if some samples reported an insufficient Δ CTs, they were equally sequenced a second time, reporting a fulfilling coverage.

NGS Sequencing

All samples were diluted to a 5 ng/ μ L concentration and then 20 ng (4 μ L) of each sample have been used for sequencing by NGS. This was carried out in the Molecular Biology laboratory of University of Catania on Illumina MiSeq platform according to the manufacturer's instructions provided in the AmpliSeq™ Cancer HotSpot Panel v2 for Illumina® (Ref. 20019161, Illumina, Inc., 92122, San Diego, CA, USA); in the end we were able to sequencing 2800 COSMIC mutations from 50 oncogenes and tumor suppressor genes. Indexes were provided with AmpliSeq™ CD Indexes, Set A for Illumina® (96 Indexes, 96 Samples) (Ref. 20019105, Illumina, Inc., 92122, San Diego, CA, USA). Denature and dilute libraries were obtained following the "Denature and Dilute Libraries Guide" protocol provided by Illumina® (Document # 15039740 v10), choosing as Loading Concentration 9 pM. Finally, sequencing was performed using the MiSeq Reagent Kits v3 (Ref. 15043895, Illumina, Inc., 92122, San Diego, CA, USA). The creation of the Sample Sheet was accomplished by using the Local Run Manager v3 software and following the instructions in the Local Run Manager v3 Software Guide provided by Illumina.

Bioinformatics Analysis

The data obtained from the sequencing were uploaded to the Illumina BaseSpace Sequence Hub platform as FASTQ format and analyzed with the "DNA Amplicon" application by Illumina, inc. for the detection of mutations in the genes belonging to the panel. Further information regarding the mutations obtained, i.e. amino acid mutations, the impact on protein structure and dbSNP_IDs, was inserted into the online tool PROVEAN®, to provide predictions for any type of protein sequence variations including the following. Clinical considerations, population incidence (MAF) and genomic information were obtained from <https://www.ncbi.nlm.nih.gov/snp/> and <https://www.ncbi.nlm.nih.gov/clinvar/>.

Multiparameter Genetic Score

For each patient we calculated the multiparameter genetic score that was obtained considering the genetic landscape, the allelic frequency spread among population and the clinical features. For each non-synonymous dbSNP sequenced and reported in literature, we created three scores that take into account: the tropism of the dbSNP, i.e. on which samples the mutation was detected (Tropic Score); the allele frequency in the population (MAF Score); its clinical significance (ClinVar Score). We calculated the three scores for each of our patients according to the specific mutation found and sequenced, in order to understand which was the factor that contributed the most to the neoplasm growth. Each score scale ranges from a minimum of 10 (less severe condition) to a maximum of 150 (more severe condition).

Once the three scores have been applied for each dbSNPs present in every single patient, three summations were calculated (one per single score $\sum TropismScoreinpatientn$, $\sum MAFScoreinpatientn$ and $\sum ClinicalScoreinpatientn$).

cBioPortal analysis

cBioPortal (<https://www.cbioportal.org/>) provides a visual tool for research and analysis of cancer gene data and helps cancer tissue and cytology research gain molecular data understanding of their genetics, epigenetics, gene expression and proteomics¹⁵. Through cBioPortal online tool, we analyzed gene alterations and their correlations with clinical features.

Statistical analysis

Continuous variables are presented as median and interquartile range (IQR) and were compared by the student independent t test or the Mann–Whitney U test based on their usual or unusual distribution, respectively (normality of variables' distribution was tested by the Kolmogorov–Smirnov test). Categorical variables were tested with the χ^2 test.

To establish a potential correlation among the three scores and the globally calculated score and other clinical and non-clinical data i.e. age, prostate specific antigen (PSA) values at the moment of diagnosis, number and spread of metastases (M), spread to lymph nodes (N), TNM Staging (pT) and Gleason Score, Pearson's correlation analyses were performed for the 17 patients; in particular, the analysis took into account both the global score, deriving from each of the finding mutation, and the specific percentage score from each of the three summation. This correlation matrix is a statistical tool that measures the linear correlation between two variables: X and Y. The matrix has a value range between + 1 and - 1, where + 1 indicates total positive linear correlation, 0 no linear correlation, and - 1 total negative linear correlation. The correlation coefficient ranges from - 1 to + 1, where + 1 implies the existence of a linear equation establishing a relationship between two factors (X and Y) that increase simultaneously. In addition to this, R-Squared was also calculated, which measures the reliability of the linear relationship between the variables included in the model. Its value is between 0 (fully correlated variables) and 1 (unrelated variables).

For all statistical comparisons, a significance level of $p < 0.05$ was considered to show any substantial difference between groups.

Data analysis was performed under the guidance of our statistics expert, using SPSS version 17 (Statistical Package for Social Science. SPSS Inc. Released 2008. SPSS Statistics for Windows, Version 17.0.) (SPSS Inc.).

Results

Table 1 lists the baseline characteristics of patients.

Table 1
Baseline characteristics of the patients

Age (years), median (IQR)	65.0 (62.0–68.0)
PSA (ng/ml), median (IQR)	15.9 (9.37-40.0)
Clinical stage, n (%)	
T1-T2	14 (82.35)
T3	3 (17.65)
Biopsy ISUP score, n (%)	
1	1 (5.88)
2	6 (35.29)
3	2 (11.76)
4	6 (35.29)
5	2 (11.76)
Pathological stage, n (%)	
T2	1 (5.88)
T3a	5 (29.41)
T3b	11 (64.71)
Pathological ISUP score, n (%)	
2	1 (5.88)
3	4 (23.53)
4	2 (11.76)
5	10 (58.82)
IQR = interquartile range; PSA = prostate specific antigen; ISUP = International Society of Urological Pathology	

DNA Amplicon Sequencing

Average Quality Parameters are reported in *Supplementary table 1*.

On average, 15 SNV/SMVs were sequenced from each sample, for a total of 688 mutations in the panel's gene hotspots and 39 nonsynonymous mutations associated with a dbSNP or COSMIC code analyzed. Furthermore, for 5 of these mutations any reference (dbSNP or COSMIC) was found in literature. However,

these 5 mutations were sequenced just once in one single patient. Moreover, these mutations were sequenced on a total of 25 genes. The distribution of the mutations analyzed is shown in Table 2.

The patient where were found most non-synonymous dbSNPs is Patient 8 (15 dbSNPs) and this is also the patient with the higher number of Somatic Mutations, sequenced only in tumor or lymph node samples (8 dbSNPs in Tumor and 2dbSNPs in Lymph node sample). The patient with the minor number of dbSNPs is patient 12, with only 3 dbSNPs and both Germline, since they have been sequenced on Mucosa and Tumor sample. The most diffused dbSNP sequenced is rs1042522 on TP53 gene (16 patients out of 17 reported this specific mutation) while the less diffused are the rs1419499014 on ERBB2 gene, the rs990046031 on AKT1 gene, the rs587781474 on STK11 gene, the rs1800863 on RET gene, the rs839541 on ERBB4 gene, the rs199906407 on FTL gene, the rs780807237 on PIK3CA gene, the rs28934578 and the rs587782006 both on TP53 gene, the rs397772062 on KDR gene, the rs34323200 on NPM1 gene, the COSM5990839 and rs121913329 both on APC gene, the rs149119664 on FGF3 gene, the rs876659657 on MLH1 and finally the rs752729752 and the rs75580865 both on FLT3 gene. All of these mutations were identified only in one sample out of 44. In order to extract not only germline variants, but also the somatic ones the fastq patients' samples were compared in pair for each patient. In fact, tumoral versus healthy tissues and lymph node versus healthy samples have been analyzed and compared.

Multiparameter Genetic Score

All three scores that are part of MGS have been calculated. The percentages of all 17 patients are reported in suppl. Table 3. Almost all the patients showed a coherent trend: the MAF score was the major component in all 17 patients (MAF score ≥ 41.5 always). The ClinVar score was the second major component ($18.3 \leq \text{ClinVar score} \leq 38.7$) in 12 patients out of 17 while the Tropism score was the minor component in almost all patient (12 out of 17, $6.7 \leq \text{Tropism Score} \leq 33.7$). The average values showed that MAF Score was the major constituent of all MGSs while the ClinVar and Tropism Scores were respectively about 1/4 (26.5%) and 1/5 (20.4%). The Percentages were not sample-dependent, since the values were heterogeneous both in patients with lymph node metastatic tumor and patient with non-lymph node metastatic ones. The Correlation Matrix and then Results of the R square test are reported in the Fig. 1. The correlation analysis performed involves clinical parameters and parameters introduced by us: consequently, it would be fallacious to define as valid only Pearson coefficients very close to +1 or -1. Consequently, all values of $-0.25 < \rho < +0.25$ indicate lack of correlation, while values of $\rho \geq +0.25$ or values of $\rho \leq -0.25$ indicate respectively positive and negative correlation. As for R-Square test, the variables that reported R values minor of 0.05 are MAF vs. Tropism Score (0.0001, Highly Correlated), MGS vs. Tropism Score (0.08, Slightly Correlated) and lymph node metastasis vs. Tropism Score (0.01, Slightly Correlated) (Table 3).

Table 3
Correlations considered as significant in our Correlation Analysis and relative Pearson Coefficient Values. N = lymph node metastasis.

Correlation	ρ Value	Correlation	ρ Value
MAF VS Tropism	-0.80	Age VS ClinVar	+ 0.36
ClinVar VS Tropism	-0.45	Age VS Gleason	+ 0.29
Score VS MAF	-0.27	pT VS ClinVar	+ 0.30
N VS MAF	-0.45	pT VS PSA	+ 0.29
N VS ClinVar	-0.46	MGS VS Tropism	+ 0.43
Age VS MAF	-0.40	N VS Tropism	+ 0.69
Score VS ClinVar	-0.30	N VS MGS	+ 0.29

Total Impact of dbSNPs on AmpliSeq Panel

A total of 688 Intronic, Synonym and Non-Synonym mutations were sequenced. These mutations were sequenced on 32 different genes (64% of the 50 genes included in the panel). The gene with the most sequenced mutations were ERBB4 (83 mutations, 12% of 688 total) while the least mutated were AKT1, FGFR2 and MLH1 (1 mutation alone, 0.14% of 688 total). The gene that reported the most nonsynonymous mutations was the TP53 gene, where a total of 42 mutations were sequenced and all nonsynonymous. Suppl. Table 4 resumes all mutations sequenced.

The relationship between ERBB4, HRAS, KIT, ABL1, CTNNB1 and prognosis

We studied those alterations that were expressed both in primary tumor and in matched lymph node metastasis.

To further understand the findings in our cohort, we used the cBioPortal online tool to study the alterations of the ERBB4, HRAS, KIT, ABL1, CTNNB1 and their correlation with prognosis using 23 studies selected (9377 samples).

ERBB4 was muted in 1.7% of cases, KIT in 1.3%, ABL1 in 1.4%, HRAS in 1.3%, CTNNB1 in 4% (Suppl. Figures 1–5).

We did not find significant association regarding overall survival and disease-free survival in positive patients at ERBB4 and ABL1 (Suppl. Figures 6–7). On the contrary, we found significant association between KIT alteration, overall survival and disease-free survival (Suppl. Figure 8), between HRAS and overall survival (Suppl. Figure 9) and between CTNNB1 and overall survival (Suppl. Figure 10).

When incorporating KIT, HRAS and CTNNB1 alteration in combination score we found that overall survival was statistically worse ($p < 0.01$) and also disease-free survival ($p = 0.02$) respect to unaltered group (Fig. 2).

Tissue Micro array analysis and immunohistochemistry stainings.

Regression analysis demonstrated weak statistical association between IGF1-R expression and ERBB4 alterations ($r = 0.005$; $p < 0.01$). Univariate logistic regression analysis showed strong association between IGF1-R expression and ERBB4 alterations in primary tumor or lymph node metastasis (odds ratio: 54.0; $p < 0.01$). For the AR, IR- α , IR- β , and PSMA expression, we did not find statistical associations.

Discussion

Herein in the present study we reported genomic alterations in primary prostate cancer tissue and matched lymph node metastasis. In particular, KIT, HRAS and CTNNB1 were associated with worse overall survival disease free survival ($p = 0.02$) respect to unaltered group in cBioPortal.

In an interesting study by Zheng et al it has been investigated the role of 37 genes to predict lymph node invasion. The results of the RNA sequence in this study showed that 18 of 37 genes exhibited dysregulated expression between PCa and lymph node invasion samples, indicating that dysregulated expression levels of different genes played an important role in the LNI of PCa¹⁶.

A similar well-designed study aimed at identifying the genes associated with the involvement of lymph nodes metastasis in patients with PCa and among 376 genes investigated three genes, RALGPS1, ZBTB34, and GOLGA1, had a significant copy number alteration¹⁷.

Pudova et al performed a bioinformatic analysis of The Cancer Genome Atlas (TCGA) data and RNA-Seq profiling of a Russian patient cohort to reveal prognostic markers of locally advanced lymph node-negative prostate cancer. Authors found different genes that were associated with favorable and unfavorable prognosis based on the TCGA (B4GALNT4, PTK6, and CHAT) and Russian patient cohort data (AKR1C1 and AKR1C3). Furthermore, authors revealed such genes for the TMPRSS2-ERG prostate cancer molecular subtype (B4GALNT4, ASRGL1, MYBPC1, RGS11, SLC6A14, GALNT13, and ST6GALNAC1)¹⁸.

Similarly, Schmidt et al we analyzed laser micro-dissected primary PC foci ($n = 23$), adjacent normal prostate tissue samples ($n = 23$) and lymph node metastases ($n = 9$) from ten hormone-naive PC patients. Genes important for PC progression were identified using differential gene expression and clustering analysis and they reported a list of 20 transcripts (2 upregulated, 18 downregulated). The seeding model significantly predicted time to BCR independently of clinicopathological variables in uni- and multivariate Cox-regression analysis in TCGA (univariate: HR 2.39, $P < 0.0001$, multivariate: HR 2.01, $P < 0.0001$)¹⁹.

Several studies have investigated the genomic characteristics of metastatic hormone-sensitive PCa (mHSPC). A recent systematic review by Van der Eecken et al was conducted on 11 studies that included 1682 mHSPC patients. A comparative analysis of gene alteration frequencies across disease states revealed a relative increase from localised to castration-resistant tumours, with noteworthy enrichment of CTNNB1 alterations in mHSPC (5%)²⁰.

In fact, patients with PCa with alterations in canonical WNT pathway genes, which lead to β -catenin activation, are refractory to androgen receptor-targeted therapies, underlying that this genomic alteration may harbor a more aggressive cancer²¹.

These results are confirmed by Isaacsson Velho that showed that the different types of Wnt- pathway mutations (inactivating APC or RNF43 mutations vs activating CTNNB1 mutations) were independently associated with higher hazard of PSA progression than Wnt wild type (aHR 2.58, p = 0.023). Despite a strong trend in the same direction, CTNNB1 mutations showed no statistically significant association with higher hazard of PSA progression (aHR 2.12, p = 0.072)²².

Generally, cancer has been linked to mutations in ERBB4 gene²³, which in this study was confirmed as a highly unstable gene. No information was found on the sequenced p.Q264* missense mutation in this gene, even if this has been already sequenced in a colon cancer cell line²⁴. Regarding CSF1R, as for the SNP rs386693509, no MAF is reported on Data Bank for this mutation, but it has been sequenced on 15 patients out of 17 in our cohort (88.2%). Even if present in literature, this mutation is very little discussed and with an unknown pathogenic impact²⁵. In the FGFR gene, rs17881656 is included in a retrospective study which tests NGS of selected cancer-associated genes in resected prostate cancer²⁶, while rs149119664 is not reported in literature, even if classified as pathogenic (score by 0.97) by the FATHMM prediction.

As concerning the analysis of prostate cancer sample and matched lymph node metastasis reported in Table 2, we found mostly concordance between both samples, underlining that the identification of alterations in primary tumor is extremely important for cancer prognosis prediction.

The Cancer Genome Atlas (TCGA) showed that 13 genes were recurrently mutated in prostate cancer: deletions of SPOP, TP53, FOXA1, PTEN, MED12, and CDKN1B; additional clinically relevant genes were identified with lower frequencies, including BRAF, HRAS, AKT1, CTNNB1, and ATM²⁷.

Despite the confirmation of these interesting results, it is important to underline that it is not easy and reliable to perform such analysis in standard patients during clinical settings, since we have also to consider the dynamic of tumor and long follow-up to be fulfilled. In this context, liquid biopsies may overcome the limitations of inpatient tumour heterogeneity and of tissue biopsies allowing for monitoring PCa disease^{28,29}.

Although there are no technology available to detect circulating tumor cells in all their phenotype and dynamic processes, however new platforms and further studies are ongoing to overcome all limitations³⁰. In this context, the analysis of common alterations that are matched with lymph node metastasis can be helpful for cancer prognosis and treatment.

Before concluding we would like to underline some limitations. Firstly, the discovery data was small since included 17 patients and 45 samples. Furthermore, we applied a standard cancer hotspot and we did not assess further alterations.

Before concluding we would like to underline some limitations. Firstly, the discovery data was small since included 17 patients and 45 samples. Furthermore, we applied a standard cancer hotspot and we did not assess further alterations.

Conclusions

In the present study we reported genomic alterations in primary tumor and matched lymph node metastasis from hormone-naïve prostate cancer patients. We revealed that the gene with the most sequenced mutations were ERBB4 (83 mutations, 12% of 688 total) while the least mutated were AKT1, FGFR2 and MLH1 (1 mutation alone, 0.14% of 688 total). We also found mostly concordance between both primary PCa samples and matched lymph node metastasis in terms of genomic alterations, underlining that the identification of alterations in primary tumor is extremely important for cancer prognosis prediction. Analyzing data from cBioPortal we demonstrated worse overall survival and disease-free survival in patients with combined alteration of KIT, HRAS and CTNNB1. These results can be applied for monitoring disease and drug response after curative treatment.

Declarations

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None

CONFLICT OF INTEREST

Each author discloses no conflict of interest.

Datasets are available on request:

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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Tables

Table 2 is available in the Supplementary Files section.

Figures

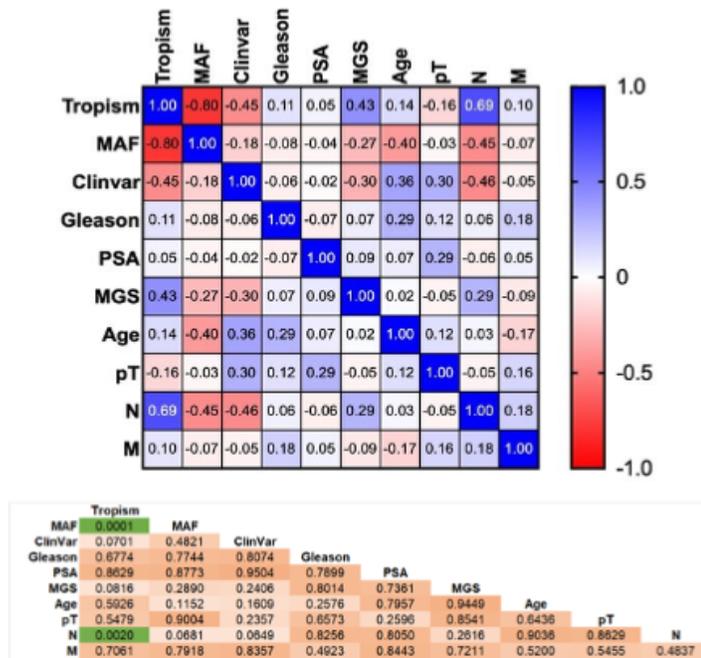


Figure 1

Legend not included with this version.

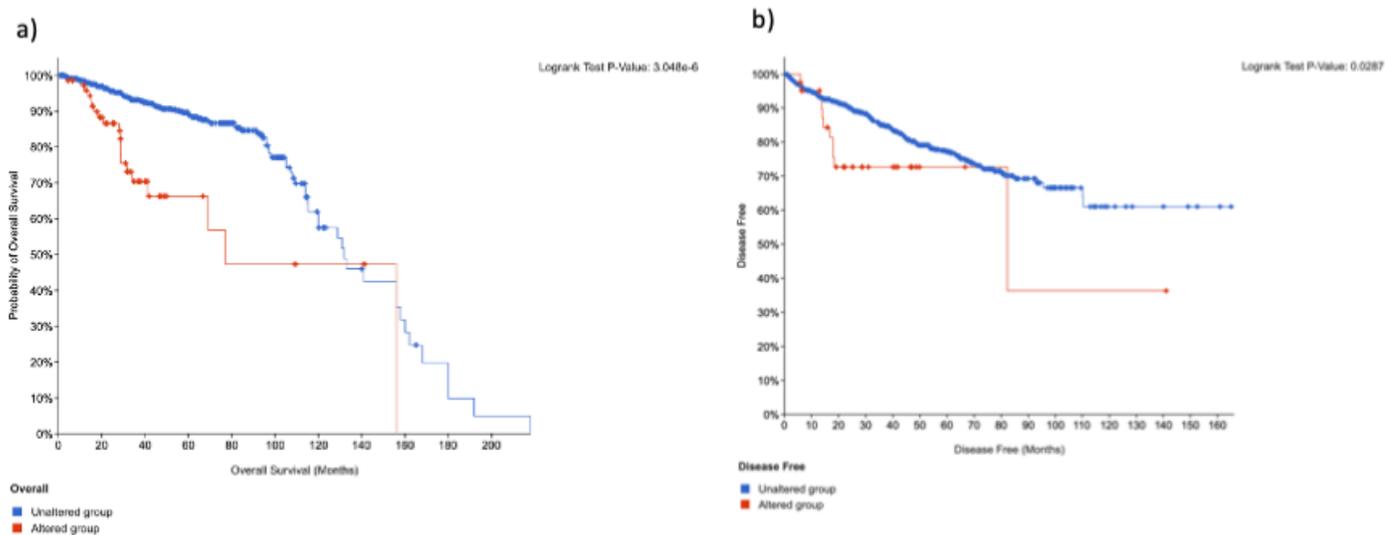


Figure 2

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