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THE ASSOCIATION BETWEEN THE IL-1 PATHWAY

Isaac C. Wun

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THE ASSOCIATION BETWEEN THE IL-1 PATHWAY AND MELANOMA OUTCOMES

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A

DISSERTATION

Presented to the Faculty of

The University of Texas

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Graduate School of Biomedical Sciences

in Partial Fulfillment

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for the Degree of

DOCTOR OF PHILOSOPHY

by

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Houston, Texas

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THE ASSOCIATION BETWEEN THE IL-1 PATHWAY

AND MELANOMA OUTCOMES

Isaac Wun, B.A., M.A.

Advisory Professor: Christopher Amos, Ph.D.

Cutaneous malignant melanoma (CMM) is a potentially lethal malignancy that warrants attention and further research, as it is known to that there is an increasing rate of incidence in the United States, and it is also known that exposure to UV light is its most crucial risk factor, and family history of melanoma is also an important risk factor. Melanoma is an aggressive and lethal cancer in humans. There are an estimated new 132,000 melanoma cases annually worldwide, and the trend has doubled in the past 20 years. However, attempts to treat melanoma have encountered considerable resistance and remained ineffective. The 5-year survival rate for metastatic melanoma remains less than 5%. CMM patients may develop an immune response to their tumors, but innate anti-tumor immune responses are insufficient for controlling the development of the tumor. Melanoma is a very immunogenic tumor, sometimes exhibiting spontaneous remissions, making it one of the foremost targets for immunotherapy. Unfortunately, despite the attempts at making tumor-infiltrating lymphocyte treatments, the clinical response has been borderline. The immunosuppressive microenvironment of tumor cells becomes significant and intertwines with the resistance of tumor treatment. Furthermore, IL-1 can hinder the immune response to melanoma. The pathway of MAPK activation leads to the production interleukin(IL)-1α/β. IL-1 conducts immunomodulatory activity through tumor-associated fibroblasts and locks in the turnkey position of the immunosuppression pathway to resist the cytotoxic T lymphocyte function. Hence, IL-1 is a key target of interest in treating melanoma, along with the entire

pathway flowing from it. Polymorphisms in genes regulating the immune response could result in increased susceptibility to and/or poorer prognosis in certain individuals. For this study, one of the objectives was to examine if single nucleotide polymorphisms (SNPs) of certain pro- and anti-inflammatory cytokines and growth factors, namely IL-1, IL-6, IL-8, IFN-γ, and TNF-α are associated with melanoma outcome of death, recurrence, or composite, and thus susceptibility. Those genes are the upstream and downstream targets for the greater IL-1 pathway. The greater IL-1 pathway has multiple genes in between those upstream and downstream targets. Those genes are IL-1RI, IL-1RAcP, IL-1RA, MYD88, TOLLIP, IRAKs, MEKK1, MEK3, MEK6, JNK, P38, c-JUN, ECSIT, TRAF6, TAB1, TAK1, RKIP, NIK, IKKα, IKKβ, IκBα, and NF-κB.

The individual SNP analysis proved to be interesting though inconclusive. There were some borderline significant results, such as associations of the SNPs rs16944, rs1143627, rs1071676, and rs3136558 with the endpoint of death. The French validation verified a significant association of rs3136558 with death but none of the others.

The next objective was to perform a full pathway analysis, incorporating all available SNPs for each gene in the overall IL-1 pathway to determine if any components were significant and if so, to attempt further verification at the protein level if the data were available. By using the SKAT program for pathway analysis, several genes in the IL-1 pathway were found to be significant. They were IL-1, c-JUN, and ECSIT, with borderline significance for TAK1.

With the observation of associations of these four genes with melanoma outcomes at the DNA level, the next step was to determine if they were also significantly associated with melanoma outcomes at the expression level. The data for the DNA level studies did not

include protein expression studies, so we used data provided by The Cancer Genome Atlas (TCGA). TAK1 data was not available, but RNA expression data were gathered for IL-1, c-JUN, and ECSIT. At the protein level, only c-JUN data was available. At the RNA level, IL1 showed that survival was proportional to IL-1 level, ECSIT showed lower survival for higher level, while c-JUN showed higher survival for higher levels. At the protein level, c-JUN was significant at the protein level, both adjusted and unadjusted via logistic regression. It seems that for developing further therapy against melanoma, c-JUN may be be a crucial target in the IL-1 pathway. IL-1 and ECSIT could also play important roles related to melanoma outcomes but require future studies where protein expression data is available to confirm the DNA- and RNA-based results.

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Chapter 1

Introduction

1.1 Melanoma Lethality and Association with IL1 Pathway

Melanoma is an aggressive and lethal cancer in humans. There are an estimated new 132,000 melanoma cases annually worldwide, and the incidence has doubled in the past 20 years (Gray-Schopfer et al., 2007). However, attempts to treat melanoma have remained largely ineffective. (Ivanov et al., 2003; Hersey et al., 2006; Gray-Schopfer et al., 2007; Segura et al. 2013). There are new concepts directed at enhancing the curability of melanoma (Gray-Schopfer et al., 2007; Finger et al. 2013; Segura et al. 2013), such as targeted therapy to control immunosuppression that typically develops in metastatic melanoma, inhibition of connective tissue growth factors, epigenetic approaches against core transcriptional activities. This study intends to use a bioinformatics approach to identify the critical elements for enhancing therapeutic effectiveness.

Melanoma, as one of the most immunogenic tumors for which spontaneous immune responses and remissions can rarely occur, is one of the foremost targets for immunotherapy (Zito, 2012). Such attempts can stimulate tumor antigen-specific T-cells, but they are only effective for a few percent of individuals with metastatic melanoma (Alexandrescu, 2010).

The *IL-1* pathway plays a key role in controlling immune response and particularly upregulation of *IL-1* is associated with immunosuppression. The immunosuppression microenvironment of tumor cells becomes significant and intertwines with the resistance of tumor treatment. Khalili et al. (2013) explains that the pathway of *MAPK* activation leads to the production interleukin(*IL*)-*1α/β*. *IL-1* conducts immunomodulatory activity through tumor-associated fibroblasts and holds the turnkey position of the immunosuppression pathway to resist the cytotoxic T lymphocyte function. Qin et al. (2011) showed that

blocking the *IL-1* receptor with antibodies or siRNAs halted growth in *IL-1*-positive melanoma cells. This disruption of the *IL-1* pathway also boosted autophagy in the *IL-1*-positive melanoma cells. Hence, without such disruption, *IL-1* dampens the immune response. These results suggest that *IL-1* might play an important role in the etiology and treatment of melanoma, and thus *IL-1* becomes the main variable in this bioinformatical study, along with the entire pathway flowing down from it.

Figure 1 presents the greater *IL-1* pathway.

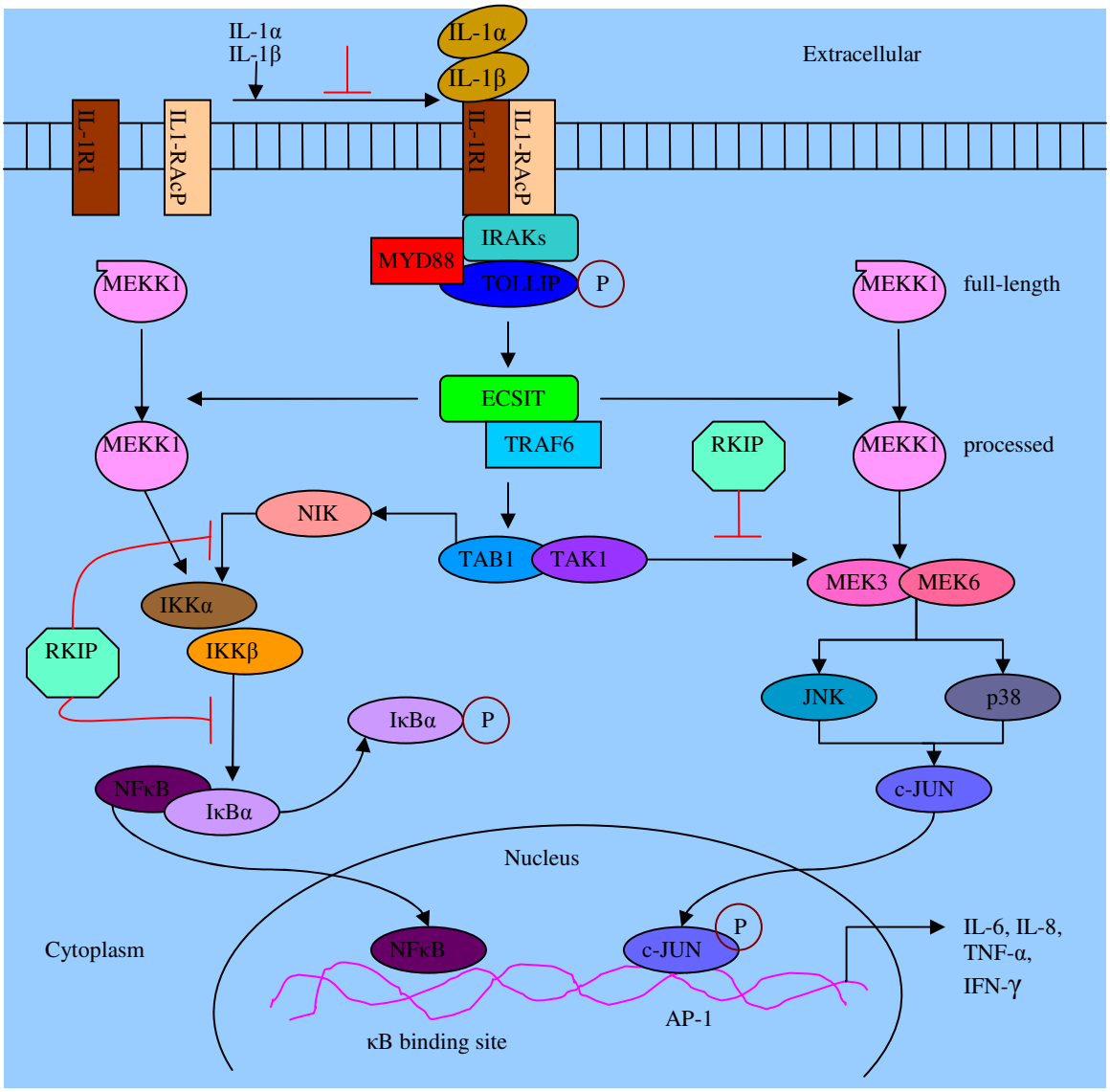


Figure 1. This figure displays the full *IL-1* pathway from the *IL-1* initiators to the final downstream targets of *IL-6*, *IL-8*, *IFN- γ*, and *TNF-α*

Khalili et al. (2013) shows that melanoma can by exacerbated by *IL-1* and other cytokines, critical components of the immune system. (Botella-Estrada, 2005) offers insights into the details of how these cytokines interrelate. Cytokines are small soluble glycoproteins generated by multiple types of cells in all human organs. Their primary purpose is to fulfill

communication among cells by binding to particular receptors. Cytokines regulate cell differentiation and growth in the immune system. Among T-helper cell (Th) subsets, Th1 and Th2 subsets originate from naïve T (Th0) cells. Th0 cells are T cells that have differentiated in bone marrow and undergone the thymal selection process. Each subtype is characterized by its own particular cytokine pattern. Several cytokines emerge from each type, but interferon-gamma (*IFN-γ*) is the most characteristic cytokine for Th1. Khalili et al. (2013) explains that Th1 and Th2 embody different sorts of immune responses. Th1 cells stimulate a delayed-type of hypersensitivity response. Th2 cells, on the other hand, incite humoral immunity. In order to maintain a well-regulated physiological state, there must be a balance between the two arms. That balance is maintained by *IFN-γ* which inhibits Th2 cells, while interleukin(*IL*)-*4* and interleukin(*IL*)-*10* inhibit Th1 cells. Dendritic cells also maintain a vital function in the formation of an effective immune response, since they are the most powerful antigen-presenting cells.

(Boyano, 2000) observed that cytokines are not only secreted by normal cells. Tumor cells also secrete cytokines, and one can expect the tumor versions to be deranged. Martinez-Escribano, 2002 observes that various cytokines can exhibit opposite effects upon the growth of tumor cells, with examples being *IL-10*, interleukin-6 (*IL-6*), and *IFN-γ*. Martinez-Escribano, 2002 goes on to explain that the outcome for melanoma can be difficult to predict, particularly when taking into account that the tumor cells interact in complicated ways with the immune system. *IL-6* and *IFN- γ* can both either hinder proliferation of melanoma cell or accelerate the growth rate, depending on the melanoma stage. It is logical that analyzing *IL-6* and *IFN-γ* gene polymorphisms could help researchers to better understand the function of those cytokines in the immune response against melanoma cells. The results for the study

indicated that *IL-6* might display opposite effects on melanoma cells, being an inhibitor for early stage melanomas and as a mitogen at more advanced stages of the cancer. It seems that *IL-6* serums levels are higher and correlated with major tumor burdens along with lower survival rates. Martinez-Escribano, 2002 could not demonstrate a correlation between *IFN-* γ medium and/or high expression genotypes and a worse prognosis.

All the previously mentioned cytokines are affected by *IL-1* as the initiator, since they are all downstream targets in the *IL-1* pathway. Thus, genetic variation in immune-regulating components such as cytokines, specifically of the *IL-1* pathway, may lead to differences among individuals in immunosuppression response and susceptibility to melanoma. Therefore, this study is warranted in order to better identify which components of the *IL-1* pathway are crucial target for improving treatment of melanoma. Due to the potential opposite functions of *IL-1*, genetic variation in immune-regulating components, and microenvironment variation, the study of melanoma tumorigenic mechanisms is a very complex matter. It may be due to these complicated mechanisms that the treatment of melanoma is ineffective. To further elucidate this complex mechanism, this study intends to examine the relationship between gene activity of upstream and downstream targets for *IL-1* pathway, (i.e. *IL-1*, *IL-6*, interleukin(*IL*)-*8*, *IFN-*γ, and tumor necrosis factor-alpha (*TNF-*α)), correlation with the progression of melanoma using the bioinformatical approach.

To further describe the downstream targets of the *IL-1* pathway and their association with melanoma outcomes, various cytokines can exhibit opposite effects upon the growth of tumor cells, with examples being interleukin-10 (*IL-10*), interleukin-6 (*IL-6*) and interferon-gamma (*IFN-*γ). Martinez-Escribano, 2002 notes that the outcome for melanoma can be difficult to predict, particularly when taking into account that the tumor cells interact in

complicated ways with the immune system. *IL-6* and *IFN-γ* can both either hinder proliferation of melanoma cell or accelerate the growth rate, depending on the melanoma stage. They may encourage proliferation for advanced melanoma while inhibiting progression at the early stages. It is logical that analyzing *IL-6* and *IFN-γ* gene polymorphisms could help researchers to better understand the function of those cytokines in the immune response against melanoma cells. The results for the Martinez-Escribano study indicated that *IL-6* might display opposite effects on melanoma cells, being an inhibitor for early stage melanomas and as a mitogen at more advanced stages of the cancer. *IL-6* serums levels are higher and correlated with major tumor burdens along with lower survival rates. The study could not demonstrate a correlation between medium and/or high expression genotypes for *IFN-γ* and a worse prognosis. (Martinez-Escribano, 2002) Another downstream target of the *IL-1* pathway is *IL-8*. *IL-8* could be associated with the potential for metastasis. It was noted by Singh (1995) that there were no detectable levels of mRNA transcripts in the majority of melanoma cases, only in the highly metastatic one. *IL-1* upregulates *IL-8* and thus induces its production.

Earlier studies did find elevated *IL-6* levels in melanoma patients. The paper noted that those studies had subjects with advanced-stage melanoma. As to why the levels of cytokines had been changed, some possibilities were cytokine production by the metastases, cytokine production by lymphocytes in response to the tumor, and perhaps there was a genetic factor regarding polymorphisms. (Porter, 2001)

TNF-α is a cytokine that is produced in several types of cells, though mostly in mononuclear phagocytes. Cytotoxicity and anti-tumor activity stand amongst its various functions. As such, it bears a critical role in immune responses, along with proliferation and

inflammation. However, if the level reaches excess, there can be toxic systemic effects. (Ocvirk, 2000)

1.2 Therapeutic Difficulties and Potential

Despite the immense potential of immunotherapies for cancer, a persistent barrier has been the obstruction posed by complexities in the tumor microenvironment, with agents and cells secreting immunosuppressive effects. The mechanisms for initiating and maintaining immunosuppression in neoplastic lesions to permit tumor growth is a matter that remains to be explained. The article by Khalili et al. (2013) noted that immunosuppression can be initiated by the upregulation of v-raf murine sarcoma viral oncogene homolog B (*BRAF*), mutated to the active form, *BRAFV600E*, in more than half of melanoma patients. They noted *BRAFV600E*-induced transactivation of genes coding for both interleukin-1α (*IL-1α*) and interleukin-1β (*IL-1β*) in melanocytes and wild-type *BRAF*-expressing melanoma cells. However, the results did not display a clear correlation between the V600E mutation and the production of those cytokines.

As noted before, *IL-1* is a pleiotropic cytokine that is expressed by many tumor types. With various models, *IL-1* shows either protumor or antitumor functions. Khalili et al. (2013) discusses that the results seem to indicate that the oncogene driven activation of the *MAPK* pathway can induce tumor cells to secrete factors that modulate the immune response. *MAPK* include *p38* and *JNK*, which stimulate *c-JUN*. Among those factors, *IL-1* by itself can induce a sequence for the immunosuppressive activity of tumor-associated fibroblasts (TAFs). It is indicated that oncogene activation has a significant effect on the tumor microenvironment, and multiple factors are involved. Furthermore, the effect seems shifted more towards the

side of immunosuppression. Khalili et al. (2013) observes that Anakinra and other such clinical drugs that block *IL-1* are already available, and there are already clinical trials in cancer patients currently being conducted to test the efficacy of this drug for standalone intervention. There are no results yet from those trials. It was also noted that *IL-1* can directly encourage the growth and survival of melanoma cells, one of its pro-tumor functions. Therefore, there is both sound logic and strong incentive to focus on *IL-1* in the melanoma tumor microenvironment as a clinical target.

Melanoma being so closely tied to the immune system means that there is considerable potential for immunotherapy, which is all the more reason to study the cytokine polymorphisms in order to better refine such potential treatments regimens. This possibility has been considerably explored for human malignant melanoma (Zito, 2012). One major reason for that is because melanoma overall seems rather resistant to chemotherapy and radiotherapy (Pak, 2001).

To further the idea, while one of this study's hopes is to clarify potential targets for chemotherapy, immunotherapy is a viable alternative method for treating late-stage melanoma. There is still much to be learned, such as why melanomas become resistant toward an immune system that has been sensitized towards it. There are some potential mechanisms regarding tumor resiliency even when facing tumor-reactive T cells. Specifically, the absence of tumor-associated antigens (TAA) or HLA molecule expression might allow some melanomas to develop a stealth field of sorts that masks tumor cells from immune system recognition. Garrido F, Algarra I. (2001) noted that 63% of melanoma display loss of HLA class I expression. Mocellin, 2001 speculates that there is a possibility that other mechanisms in the tumor micro-environment, such as secretion of immune-

suppressive cytokines from the tumor, might affect immune responsiveness. (Mocellin, 2001). As an example, Estrozie et al. (2014) reported the upregulation of *BRAF* in vertical growth of cutaneous melanoma in young patients. For older patients, *BRAF* mutation may not be the dominant factor for the tumor genesis of melanoma. Khalili et al. (2013) suggested that the *BRAF* (mutated active form $BRAF^{V600E}$) activation of the mitogen-activated protein kinase (*MAPK*) pathway enhances the production of a number of immunosuppression factors by tumor cells.

Thus far, there has been no statistically significant difference between subjects receiving tumor vaccines as compared with those receiving a placebo. Leong, 2002 discusses that Th1 cells secrete IL-2, and *IFN-γ* and play a major role in generating cell-mediated immunity such as delayed hypersensitivity reaction and the generation of cytotoxic T cells, which are important for destroying cancer and virally infected cells. Despite the fact that Th1 and Th2 cells are defined by specific cytokines configurations, their interactions with one another are complex. Furthermore, it must be stressed that relationship between specific antigens and the associated cytokines produced is not completely understood, and when it comes to cancer immunology, that is doubly so.

The hypothesis for this study is that the SNPs and genes of the *IL-1* pathway should show an association, protective or detrimental, with melanoma outcome. Identifying genes that are involved in melanoma formation and progression may suggest chemoprevention or immunotherapy targets or allow for identification of groups with high risks.

1.3 Overview of Assessment Levels and Results

Specifically, in order to better understand the genetic factors that might alter the disease risk level, the purpose of the study is to assess the association between potential gene

sequences with the progression of melanoma based on the following three major endpoints: death, recurrence, and the composite of death and/or recurrence.

This relationship was assessed at the following levels:

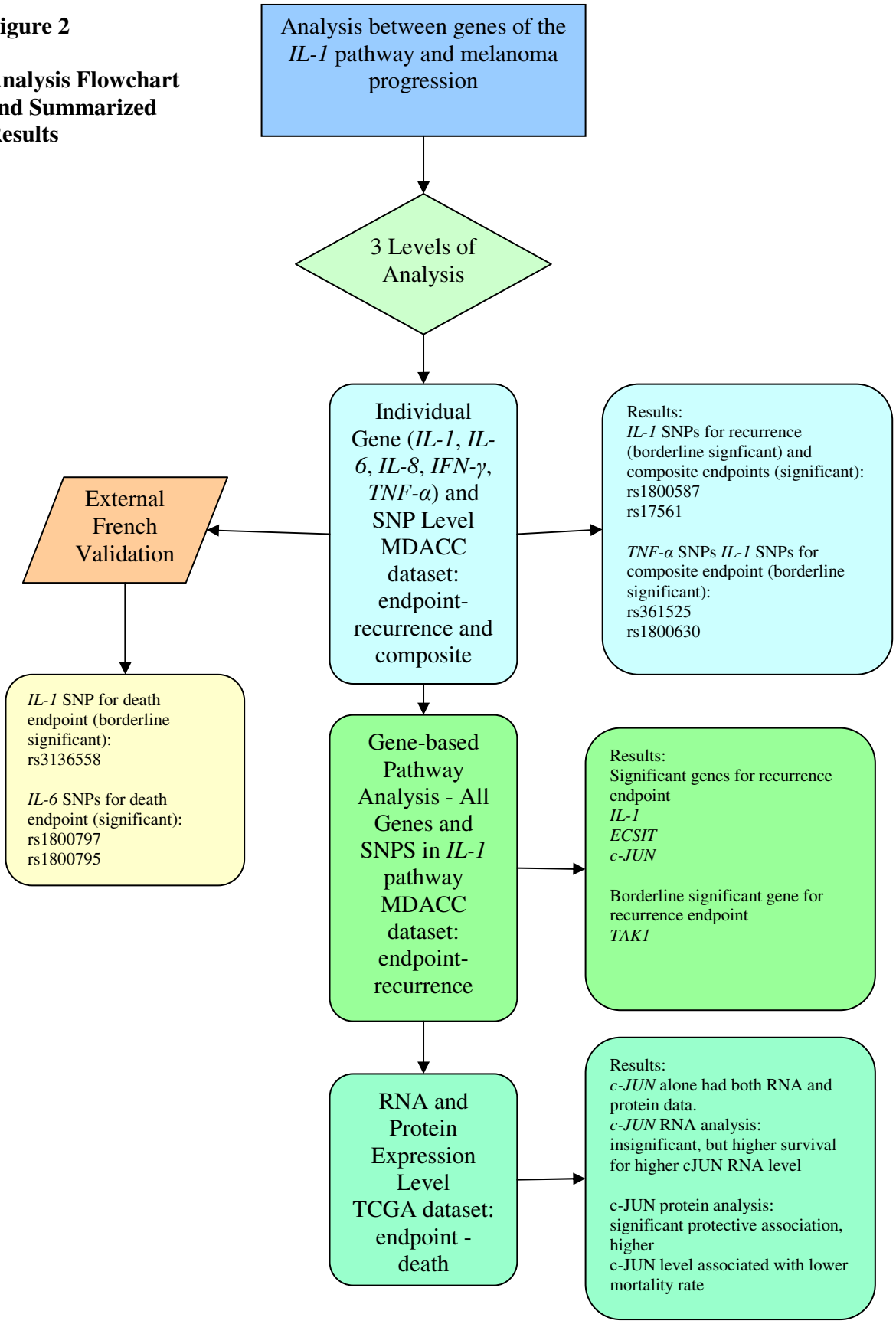
a) Individual SNP level for a list of candidate genes with external validation
b) Gene level analysis, including all available SNPs for all pathway genes
c) Those genes found to be significant from the full pathway analysis were also verified at the
    - RNA expression level, and
    - Protein expression level

Figure 1 presents a flowchart summarizing these three levels of analysis and the corresponding results. It can be referred to the methods and results sections. Identifying genes that are involved in melanoma progression may suggest chemoprevention or immunotherapy targets or allow for identification of groups with high risks.

Figure 2. Figure 2 is an analysis flowchart that summarizes the results.

Figure 2

Analysis Flowchart and Summarized Results



Chapter 2

Materials and Methods

The detailed methodologies are described below for each level of the analysis listed above in the introduction section. Figure 1 presents a flowchart of the process.

2.1 Individual SNPs Analysis with External Validation

The relationships between individual SNPs on the studied genes with melanoma outcomes of death, recurrence, and composite outcome of death and/or recurrence were assessed for the following upstream and downstream targets for the *IL-1* pathway, i.e. *IL-1*, *IL-6*, *IL-8*, *IFN-γ*, and *TNF-α*. There were a total of 20 individual SNPs that were assessed. The specific SNPs analyzed for *IL-1* were rs1800587, rs17561, rs2071374, rs16944, rs1143627, rs1071676, rs1143634, and rs3136558. For *IL-6*, the SNPs were rs1800795 and rs1800797. For *IL-8*, the analyzed SNPs were rs4073, rs2227307, and rs2227306. For *IFN-γ*, the SNPs were rs2430561, rs1861494, rs2069705. For *TNF-α*, the SNPs were rs361525, rs1799964, rs1800630, and rs1800629. The gene activity was assessed by 0, 1, and 2 copies of the ancestral allele types. The source data for this analysis includes cases (n=1804) that were drawn from a case-control study of malignant melanoma (n=931) and a case-series (n=873) all collected at the University of Texas MD Anderson Cancer Center (MDACC). All cases were required to have cutaneous malignant melanoma. Samples were genotyped by the Center for Inherited Disease Research using the Omni 1M Quad V1-0_B SNP chip. Quality control procedures were implemented at MDACC and at the University of Washington through GENEVA. During quality control processing, individuals were removed who had estimated identity by descent > 0.15 (2 or more alleles would be identical by descent if they originate from the same ancestral allele without recombination), or who failed standard QC

using PLINK (http://pngu.mgh.harvard.edu/~purcell/plink/) or Eigenstrat. DNA segments are identical by state (IBS) for 2 or more individuals if the nucleotide sequences are identical in that segment. An IBS segment is in turn identical by descent (IBD) for 2 or more individuals if it is inherited from a common ancestor without recombination. After filters for Hardy-Weinberg equilibrium ($p>0.0001$), minor allele frequency > 0.05, and missing genotypes <0.05, there were 818,237 SNPs available for analysis. The lambda value without principal component correction after excluding SNPs with minor allele frequency < 0.05 was 1.01, which decreased to 1.009 after correcting for the first two principal components. The lambda value represents the genomic control inflation factor. It is the ratio of the median of the observed distribution of the test statistic to the expected median, embodying the extent of bulk inflation and false positive rate. A lambda value above one would indicate population or genotyping errors.

Demographic and clinical data were also captured in the MD Anderson database, including ethnicity, age of diagnosis, gender, Clark level, tumor stage, and Breslow index (more information about the database can be found in the e-book 60 Years of Survival Outcomes at The University of Texas MD Anderson Cancer, chapter 2 by Sarah H. Taylor). These variables were taken into account as covariates in the analysis. Ethnicity is not an issue, as all the subjects are white. Age of diagnosis and Breslow index were treated as continuous variables, while tumor stage and Clark level were categorized into the two following groups as binary variables respectively: tumor stage III & IV vs. I & I/II; Clark level 4 & 5 vs. level 1, 2, & 3. For each of the three melanoma endpoints, time to event from first diagnosis date to the first occurrence of event date was calculated in months. The analysis data set was prepared in comma-separated values (CSV) format by combining

clinical data with genotype data for each test subject. The relationships between genotype and time to each of the three melanoma outcomes were assessed by Cox proportional hazard (PH) regression analysis (both unadjusted and adjusted). A final multivariate Cox PH model was established by stepwise regression analysis. The hazard ratios (HR) and 95% confidence intervals (CI) for per ancestral allele copy decrease were calculated to assess whether there was a protective or harmful association with the event rate for each of the three melanoma endpoints. These analyses were carried out by Statistical Analysis Software (SAS) Version 9.2.

Validation was also carried out for the individual SNP analysis in France lead by Dr. Florence Demenais, Directrice d'Unité : DR1 Inserm, of l'Université Paris Diderot - Paris 7, l'Institut Universitaire d'Hematologie. They analyzed the same 20 SNPs of the 5 genes focused upon for this study, or the closest available SNPs if the specified ones were not available. Using their own data collected from the French MELARISK cohort including 1179 melanoma cases, they also adjusted for the same set of covariates of ethnicity, age of diagnosis, gender, Clark level, tumor stage, and Breslow index. Ethnicity was not an issue, as all were of western European descent. Dr. Demenais' group also used a multivariate Cox PH model established by stepwise regression analysis. Recurrence data was not available for them, so they used the endpoint of death only.

2.2 Full Gene-Based Pathway Analysis

Given the limited power presented by single marker association studies, especially rare variants, a pathway analysis including available SNPs for all the genes in the *IL-1* pathway was necessary. Besides the sole upstream target, *IL-1*, and the downstream target of

IL-6, *IL-8*, *IFN-γ*, and *TNF-α*, this pathway analysis also include the following genes in the middle of *IL-1* pathway: *IL-1RI*, *IL-1RAcP*, *IL-1RA*, *MYD88*, *TOLLIP*, *IRAKs*, *MEKK1*, *MEK3*, *MEK6*, *JNK*, *p38*, *c-JUN*, *ECSIT*, *TRAF6*, *TAB1*, *TAK1*, *RKIP*, *NIK*, *IKKα*, *IKKβ*, *IκBα*, and *NF-κB*.

For the pathway analysis, the sequence kernel association test (SKAT) package for R was utilized. The variables of age of diagnosis, gender, Clark level, stage, and Breslow index were adjusted for as covariates. As per Wu et al. (2011) [11], SKAT is a supervised test meant for examining the joint effects of multiple variants upon a phenotype in a region. Regions are defined by using genes (in candidate gene or whole exome studies) or moving windows across the genome (in whole genome studies). For each such region, SKAT will calculate a p-value for association while adjusting for covariates. Adjustments for multiple comparisons are necessary for analyzing multiple regions, e.g. using the Bonferroni correction or false discovery rate (FDR) control. Genes were defined with specific windows per the GRCh37 build. They would be as follows:

IL-1α:

Chromosome 2; 113531492 to 113542971, complement

IL-1β:

Chromosome 2; 113587337 to 113594356, complement

IL-6:

Chromosome 7; 22766766 to 22771621

IL-8:

Chromosome 4; 74606223 to 74609433

IFN-γ:

Chromosome 12; 68548550 to 68553521, complement

TNF-α:

Chromosome 6; 31543344 to 31546113

As for notation, as per the paper by Wu et al., one would assume n subjects are sequenced in a region with p variant sites observed. Covariates might include such factors as age and gender. For the i-th subject, y_i denotes the phenotype variable, $\mathbf{X_i}=(X_{i1}, X_{i2}, .., X_{im})$ the covariates, and $\mathbf{G_i}=(G_{i1}, G_{i2}, \ldots, G_{ip})$ the genotypes for the p variants within the region. One would make the assumption of an additive genetic model and let $G_{ij,}$=0, 1, or 2 represent the number of copies of the minor allele. Regarding how SKAT operates, using the example in the paper, one would test for the relationship between genetic variants and phenotype with multiple linear and logistic regression. One would use the following linear model:

$y_i = \alpha_0 + \boldsymbol{\alpha}'\mathbf{X_i} + \boldsymbol{\beta}'\mathbf{G_i} + \varepsilon_i$

for phenotypes that are continuous traits. The following logistic model would be used for dichotomous traits such as outcome of case or control (y = 0/1 for case/control):

$\text{logit } P(y_i = 1) = \alpha_0 + \boldsymbol{\alpha}'\mathbf{X_i} + \boldsymbol{\beta}'\mathbf{G_i}$

α_0 would be the intercept term, $\boldsymbol{\alpha}=[\alpha_1,..., \alpha_m]'$ is the vector of regression coefficients for the m covariates, $\boldsymbol{\beta}=[\beta_1,\ldots,\beta_p]'$ is the vector of regression coefficients for the p observed gene variants in the region, and for continuous phenotypes ε_i stands for the error term with mean zero and variance σ^2. With both the linear and logistic models, analyzing if the gene variants influence the phenotype, adjusting for covariates, corresponds to testing the null hypothesis H_0: $\boldsymbol{\beta}=\mathbf{0}$, i.e. $\beta_1=\beta_2=\ldots=\beta_p=0$. The typical p-DF likelihood ratio test would have little power, especially in regards to rare variants. Therefore, to increase the power, SKAT tests H_0 by assuming each β_j follows an arbitrary distribution with mean zero and variance $w_j\tau$, with τ

being a variance component and *wj* a pre-specified weight for variant *j*. With the null hypothesis of H_0: **β=0**, this test is equivalent to testing H_0: $\tau=0$. A variance component score test can be constructed in the following mixed model, which is known to be a locally most powerful. One crucial advantage of this score test is that it only requires fitting the null model $y_i=\alpha_0+\boldsymbol{\alpha}_1'\mathbf{X}_i+\varepsilon_i$ for continuous traits and logit $P(y_i=1)= \alpha_0+\boldsymbol{\alpha}_1'\mathbf{X}_i$ for dichotomous traits.

The variance component score statistic is:

$Q = (y - \hat{u})' K(y - \hat{u})$

where, **K=GWG'**, $\hat{\mathbf{u}}$ is the predicted mean of ***y*** under H_0, i.e. $\hat{\mathbf{u}} = \hat{a}_0 + \mathbf{X}\hat{\mathbf{a}}$, for continuous traits and $\hat{\mathbf{u}} = \text{logit}^{-1}(\hat{a}_0 + \mathbf{X}\hat{\mathbf{a}})$ for dichotomous ones. $\hat{a}_0$and $\hat{\mathbf{a}}$ are estimated under the null by regressing **y** on only the covariates **X. G** is a $n\times p$ matrix with the (i,j)-th element being the genotype of variant *j* of subject *i*, and **W=**diag($w_1,\ldots, w_p$) contains the weights of the *p* variants. **K** is an $n\times n$ matrix with (i,i')-th element equal to $K(G_i, G_{i'})$ being the summation of j = 1 to p for $w_j G_{ij} G_{i'j}$. K(,) is the linear kernel function and $K(G_i, G_{i'})$ measures the similarity between subjects i and i' in the region using the p markers. This form of K(,), as just demonstrated by $K(G_i, G_{i'})$, is called the weighted linear kernel function. Each weight w_j is specified beforehand by utilizing the genotypes, covariates, and external biological information, i.e. without using the outcome. It reflects the relative contribution of the *j*-th variant to the score statistic: if w_j is close to zero, the *j*-th variant makes only a small contribution to *Q*. Therefore, down-weighting non-causal variants and up-weighting causal variants could yield improved power. In practice, one does not know which variants are causal, so one would set $\sqrt{w_j} = \text{Beta}(MAF_j; a_1, a_2)$, which would be the beta distribution density function with pre-specified parameters a_1 and a_2 evaluated at the sample minor-allele frequency (MAF) (across

cases and controls combined) for the *j*-th variant in the data. The Beta density has a distinct advantage in being flexible and able to accommodate a wide spectrum of scenarios.

SKAT is computationally very efficient. In simulation studies, the length of time to a convergent solution depends on the sample size and the number of markers. For 300kb, 3Mb, or 3 Gb (the entire genome) on 1000 individuals, the lengths of time were 2.5 seconds, 25 seconds, and 7 hours, respectively. For the purposes of this study, calculations take seconds or a several minutes at worst. Thus, one can see the advantage of this method.

SKAT performs a region based testing like burden tests. However, as mentioned above, SKAT is a supervised method, so it directly performs multiple regression of a phenotype on genotypes for all variants in a region while adjusting for covariates. Thus, as with conventional multiple regression models, neither directionality nor magnitudes of the associations are assumed beforehand. They are estimated from the data. SKAT has greater power than competing burden tests over a range of genetic models. SKAT performs a score-based variance component test, and the calculation only requires fitting the null model by regressing phenotypes on covariates alone and computing p-values analytically. This regression framework is rather flexible, and the kernel can be adjusted to allow for epistatic effects. SKAT can be applied to any SNP set. Possible phenotype would include outcomes such as longitudinal, multivariate, and survival data. The survival data for this study is fit through the linear model used for continuous outcomes, as the phenotypes are survival time to outcome. In other words, SKAT can be applied to a wide range of sequencing-based association studies. Also, because the p-value can be obtained directly without permutation, SKAT allows for rapid calculation of the p-values in exome and genome-wide sequencing studies.

2.3 RNA Expression and Protein Expression Analysis

Those genes found to be significant from the full pathway analysis were also verified at the RNA and protein expression levels, if such data was available. Among those four genes found to be significant from full gene-based pathway analysis (i.e. *IL-1*, jun proto-oncogene (*c-JUN*), evolutionarily conserved signaling intermediate in toll (*ECSIT*), and transforming growth factor beta-activated kinase 1 (*TAK1*) (please see the results section), the analysis at the RNA expression level only includes *IL-1*, *c-JUN*, and *ECSIT* since *TAK1* data was not available.

The association between the RNA and protein levels and the outcome of survival in melanoma was also analyzed via SAS V9.2. The data were drawn from the Cancer Genome Atlas (TCGA), which is publicly available at the following website: https://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp. The relevant files for clinical information and genotypes for the SNPs and genes were downloaded. The variables of age of diagnosis, gender, Clark level, stage, and Breslow index were adjusted for as covariates. The TCGA melanoma data was originally derived from M.D. Anderson. I used logistic regression analysis to assess relationships due to lack of time-to-event data in TCGA dataset. The odds ratio (OR) and the corresponding 95% CI were computed accordingly.

At the protein level, only c-JUN data was available. In these analyses, c-JUN was treated both as a binary variable in one instance (grouped as being above or below the median: -1.12 relative units) and a continuous variable in other instance. The results for both are provided in the results section.

Chapter 3

Results

The results are described below for each of the aforementioned three levels of analysis. The summary of the results can also be found in the Figure 1 flowchart displayed above.

3.1 Individual SNP Analysis for Candidate Gene with External Validation

Out of 1804 melanoma cases from MDACC dataset, there were only 1543 subjects with complete data for the time to event of the three melanoma outcomes and all studied covariates . Table 1 illustrates that out of 1543 subjects with complete phenotypic data who were included in the anlaysis, 182 subjects (11.8%) died, 269 subjects (17.4%) experienced recurrence, and 310 subjects had the composite event of either death and/or recurrence (20.1%). The summary statistics for each of the studied covariates (i.e. gender, age of diagnosis, Clark level, tumor stage, and Breslow index) are also presented in Table 1 for the subgroup of subjects with or without the event for each of the three studied endpoints. In general, for each of the studied endpoints, the percentage of males was higher than females for subjects with event of death and/or recurrence compared to those without the event. Subjects with events were also older with higher Clark level, more advanced tumor stage, and higher Breslow index than those without events.

It was found that none of the 20 studied candidate SNPs were significantly related to time to death. For the endpoint of recurrence, after adjusting for age at diagnosis, tumor stage (III & IV vs. I & I/II), and Clark level (4 & 5 vs. 1, 2, & 3) in the final model, Table 5.2 suggests borderline significant protective effect for per one ancestral allele copy decrease of *IL-1* SNPs rs1800587 (HR [95% CI] = 0.836 [0.694, 1.008], p = 0.061) and rs17561 (HR

[95% CI] = 0.835 [0.693, 1.007], p = 0.0588) on the endpoint of recurrence. Adjusting for the same set of covariates in the final model, these two same *IL-1* SNPs were significant for the composite endpoint of death and/or recurrence, as shown in Table 6.2 with rs1800587 (HR [95% CI] = 0.823 [0.691, 0.979], p = 0.0283) and rs17561 (HR [95% CI] = 0.821 [0.690, 0.978], p = 0.027).

Adjusting for the same set of covariates in the final model (i.e. age at diagnosis, tumor stage (III & IV vs. I & I/II), and Clark level (4 & 5 vs. 1, 2, & 3)), among the four studied SNPs from *TNF-α*, two had a borderline significant association with the composite endpoint at borderline (Table 6.2). They were rs361525 (HR [95% CI] = 0.707 [0.481, 1.040] per one ancestral allele copy decrease, p = 0.0784) and rs1800630 (HR [95% CI] = 1.206 [0.976, 1.491] per one ancestral allele copy decrease, p = 0.0825). However, per one ancestral allele copy decrease of rs361525 seems to provide a protective effect with HR < 1 and rs1800630 seems to provide harmful effect with HR > 1. Regarding the linkage disequilibrium between rs361525 and rs1800630, the percentage came to 0.62%, with a p-value of 0.0058. As for the minor allele frequencies, they were 0.0587 for rs361525 and 0.1598 for rs1800630. Table 7.3 for interaction analysis showed borderline significant interaction between rs361525 and rs1800630 with a p-value of 0.0652. The nature of the interaction of the major alleles for rs361525 and rs1800630 is directional and antagonistic.

These results were compared to the data from Dr. Florence Demenais' group. As mentioned in the methods section, only death outcome data was included in this validation analysis as recurrence data was unavailable. Out of 1179 melanoma cases from the French dataset, there were only 859 subjects with complete data for the time to death and all studied covariates. Out of 859 subjects, 91 subjects (10.6%) died. The mean (SD) of age at first

diagnosis was 46.1 (17.9) years of age. This age was lower than that for the MD Anderson dataset, and the genders were more evenly distributed with 55.4% for females. The Breslow index mean (SD) was 1.5 (2.07). For the Clark level, only 29.9% were in the high level category of 4 and 5. The Breslow index and percentage of high Clark level were much lower in the French dataset than in the MD Anderson dataset. The French data presented in Table 8 showed a borderline association of rs3136558 of *IL-1* with time to death (HR [95% CI] = 0.685 [0.011, 42.803], per one ancestral allele copy decrease p = 0.072). Considering the wide variation of the hazard ratio, this result is similar to what we found for the insignificant association between these SNPs with the death outcome in the analysis of data from MDACC. In general, our results were matched with the findings obtained from Dr. Demenais' French dataset. The only discrepancy between our findings and the French results are the two following studied SNPs for *IL-6*: rs180797 and rs1800795. For these two *IL-6* SNPs, the mortality rate was significantly increased by per one ancestral allele copy decrease of rs1800797 (HR [95% CI] = 1.495 [1.116, 2.002] p = 0.00683), and rs1800795 (HR [95% CI] = 1.501 [1.127, 1.998], p = 0.00545), while our results were not significant for these two *IL-6* SNPs, being rs1800795 (HR [95% CI] = 0.984 [0.795, 1.218] p = 0.8823) and rs1800797 (HR [95% CI] = 1.017 [0.823, 1.256] p = 0.8779). While rs1800797 hazard ratios for the MDACC and French data were in the same direction, the hazard ratios of rs1800795 for the MDACC data were not. I will discuss possible explanations for these differences in the discussion section.

3.2 Pathway Analysis

Table 9 presents the significant results based on the pathway analysis. All the other genes described in the methods section that yielded non-significant results are reported in table 10, along with the total number of SNPs included in the SKAT analysis. Several genes in the *IL-1* pathway found to be significant via the SKAT pathway analysis. Overall, significant correlation was found between *IL-1* and recurrence p = (0.035). Significant correlations were also found between *ECSIT* and the outcome of recurrence (p = 0.02). Specifically, *c-JUN* was found to be highly correlated with recurrence (p = 0.008). Finally, there was borderline significant correlation between *TAK1* and recurrence (p = 0.074).

3.3 RNA Expression and Protein Expression Analysis

To further confirm the DNA results found to be significant from the pathway analysis described above, the RNA and protein level analysis was performed based on TCGA dataset.

RNA level data was available for *IL-1*, *ECSIT*, and *c-JUN*. For the RNA level analysis, out of 305 subjects from the TCGA dataset, 194 subjects with non-missing data for all studied variables were included in this analysis. The rest were missing expression outcome or information for age at diagnosis, Clark level, Breslow thickness, tumor stage, or gender. The summary statistics of demographics and baseline clinical characteristics are presented in Table 11a. The mortality rate was 43.8% (Out of 194 subjects, 85 subjects died). In general, subjects died vs. those survived were comparable for all demographics and clinical characteristics variables with the exception of Clark level, the percentage of subjects with high Clark level 4 & 5 was higher among deaths compared to survivors (69.4% vs. 52.3%).

The unadjusted and adjusted relationships between the mortality rate and each of the studied RNA expression levels as a continuous variable are shown in Table 11b and as a binary variable for RNA expression level > median vs. <= median in Table 11c respectively. Table 11b suggests no significant association between the mortality rate and each of the studied RNAs expression levels when treated as continuous variable in both unadjusted and adjusted logistic regression analysis. Similar insignificant results were found when including each RNA expression level as binary variable in Table 11c. It could just be that the RNA data was sub-optimal. The results in Table 11c illustrate that a higher survival rate was associated with a higher *IL-1* RNA level above vs. below median of 35 units (Odds ratio (OR) [95% CI] = unadjusted: 0.746 [0.422, 1.317] p = 0.3116; adjusted: 0.782 (0.428, 1.430), p = 0.4250). The opposite result was found for the *ECSIT* RNA. A lower survival rate was found for the higher *ECSIT* RNA level above vs. below the median of 670 units (OR [95% CI] = unadjusted: 1.134 [0.643, 2.000] p = 0.6643; adjusted 1.083 [0.601, 1.952] p = 0.6643). Table 11c shows a trend of higher survival for the higher *c-JUN* RNA level group, above vs. below a median of 1470 units (OR [95% CI] = unadjusted: 0.915 [0.519, 1.614] p = 0.7587; adjusted: 0.892 [0.495, 1.607], p = 0.7038). However, all results for the analysis at the RNA expression level were insignificant.

For the protein level analysis, out of 206 subjects from the TCGA dataset, 119 subjects with non-missing data for all studied variables were included in this analysis. The summary statistics of demographics and baseline clinical characteristics are presented in Table 12a. Out of 119 subjects, 55 subjects died with a mortality rate of 46.2%. In general, subjects who died were older (mean= 60.9) compared to those who survived (mean = 53.4). For all other studied variables, the two groups were comparable.

The unadjusted and adjusted relationships between the mortality rate and c-JUN of protein expression level as a continuous variable are presented in Table 12b and as a binary variable for protein expression level > vs. <= median of -1.12 units in Table 12c respectively. As opposed to the insignificant results for the *c-JUN* RNA analysis, these two tables showed significant results for c-JUN with a trend of higher survival rate for the higher c-JUN protein level (above vs. below median of -1.12 units) with an OR [95% CI] = 0.412 [0.189, 0.900] and p = 0.0262 in the unadjusted analysis and an OR [95% CI] = 0.417 [0.182, 0.957] and p = 0.0390 in the adjusted analysis (Tables 12b).

The same conclusion was found in Table 12b when c-JUN protein expression level was treated as a continuous variable. With an increment of per one unit increase in c-JUN protein expression, the OR [95% CI] = 0.405 (0.204, 0.804] with p = 0.0098 for the unadjusted analysis, and OR [95% CI] = 0.407 (0.196, 0.844] with p = 0.0156 for the adjusted analysis. In these two tables, the odds ratios with the 95% CI for c-JUN protein expression level were well below 1. Unfortunately, protein-level data was not available for *IL-1*, *ECSIT*, and *TAK1* so that future analyses will be needed to evaluate these genes that were significantly associated with melanoma outcomes in the pathway analysis.

Table 1. Summary Statistics of Demographics and Vital Status for All Subjects (Total N = 2829)

	Case
Total Number of Subjects (N)	1802
Age of Diagnosis: Years	
- n[a]	1802
- Mean ± SD	52.11 ± 14.52
Months of follow-up[b]	
- n[a]	1800
- Mean ± SD	58.54 ± 57.60
Vital Status	
- Deaths: Number of Deaths / N (%)	260 / 1802 (14.43%)

[a] n denotes Number of Subjects with non-missing data

[b] Missing first diagnosis and last diagnosis dates for two subjects in the case group (Subject ID: MN0820 and MN0850)

Table 2.1 Frequency Distribution of Subjects with or without Events and Time to Event
or Censored for Endpoints:
- **Death,**
- **Recurrence,**
- **Death and/or Recurrence**

Among Cases (Total N = 1786[a])

Endpoint	Event Rate:	Months from First Diagnosis Date to Event or Censored Date[b]:		
	n (%)	Mean ± SD	Range Min – Max	Medium (IQR[c])
Alive and without Recurrence	1324 (74.13%)	52.59 ± 46.39	0.59 - 526.59	43.60 (24.14, 64.74)
1. Death only	48 (2.69%)	42.13 ± 45.99	3.48 - 233.69	28.27 (14.90, 47.95)
2. Recurrence only	202 (11.31%)	43.29 ± 40.35	1.38 - 192.59	27.81 (12.81, 61.60)
3. Death and Recurrence	212 (11.87%)	40.06 ± 76.89	1.02 - 562.00	17.71 (8.16, 37.70)
4. Death regardless of recurrence status (1 + 3)	260 (14.56%)	61.98 ± 80.35	3.48 - 625.94	39.26 (20.52, 70.42)
5. Recurrence regardless of vital status (2 + 3)	414 (23.18%)	41.64 ± 61.77	1.02 - 562.00	21.52 (9.66, 50.60)
6. Death and/ or Recurrence (1+2+3)	462 (25.87%)	41.69 ± 60.28	1.02 - 562.00	21.96 (10.35, 50.23)

[a] Among 1802 cases, only 1786 subjects were included:
- 2 Subjects were excluded due to missing first diagnosis and
- 14 subjects were excluded due to missing data for subsequent event assessment

[b] Months to Event or Censored were calculated by (the event or censored date – first diagnosis date) / 30.4375. Where censored date = maximum of last diagnosis date and vital status date

[c] IQR denotes Inter-Quartile Range (i.e. 3rd Quartile – 1st Quartile).

Table 2.2 Baseline Demographics and Clinical Characteristics by Subjects with or without Endpoint of Death, Recurrence, and the Composite Endpoint of Death and / or Recurrence among All Melanoma Cases from MDACC data (Total N = 1543[a])

Baseline Demographics & Clinical Characteristics	Endpoint = Death Event Rate = 182 / 1543 (11.8%)		Endpoint = Recurrence: Event Rate = 269 / 1543 (17.4%)		Composite Endpoint: Death and / or Recurrence Event Rate = 310 / 1543 (20.1%)		All Melanoma Cases
	Event (N =182)	No Event (N =1361)	Event (N =269)	No Event (N =1274)	Event (N =310)	No Event (N =1233)	(N =1543)
Gender: n (%)							
- Male	129 (70.9%)	775(56.9%)	175 (65.1%)	729 (57.2%)	207 (66.8%)	697 (56.5%)	904 (58.6%)
- Female	53 (29.1%)	586 (43.1%)	94 (34.9%)	545 (42.8%)	103 (33.2%)	536 (43.5%)	639 (41.4%)
Race: n (%)							
- White	182 (100%)	1361 (100%)	269(100%)	1274 (100%)	310 (100%)	1233 (100%)	1543 (100%)
Age at First Diagnosis (Years):							
Mean ± SD	59.3 ± 14.6	51.4 ± 14.3	56.5 ± 14.2	51.4 ± 14.5	57.4 ± 14.3	51.0 ± 14.3	52.3 ± 14.5
Stage: n(%)							
- I & I/II	39 (21.4%)	913 (67.1%)	66 (24.5%)	886 (69.5%)	83 (26.8%)	869 (70.5%)	952 (61.7%)
- III & IV	143 (78.6%)	448 (32.9%)	203 (75.5%)	388 (30.5%)	227 (73.2%)	364 (29.5%)	591 (38.3%)
Clark level: n(%)							
- 1, 2, & 3	51 (28.0%)	769 (56.5%)	67 (24.9%)	753 (59.1%)	88 (28.4%)	732 (59.4%)	820 (53.1%)
- 4 & 5	131 (72.0%)	592 43.5%)	202 (75.1%)	521 (40.9%)	222 (71.6%)	501 (40.6%)	723 (46.9%)
Breslow Index:							
Mean ± SD	4.03 ± 3.82	1.63 ± 2.15	3.55 ± 3.37	1.57 ± 2.16	3.61 ± 3.66	1.49 ± 1.93	1.92 ± 2.53

[a] Among 1804 cases, only 1543 subjects with complete data were included.

Table 3.1 Relationship between Age at First Diagnosis and Time to Event or Censored for Endpoints:

- **Death,**
- **Recurrence,**
- **Death and/or Recurrence**

Among Cases (Total N = 1786[a])

Endpoint	Age at First Diagnosis:				Per one Year increase of Age at First Diagnosis:	
	Subjects with Event:		Subjects without Event:			
	n	Mean ± SD	n	Mean ± SD	Hazard Ratio (95& CI)[b]	p-Value[b]
1. Death regardless of recurrence status	260	57.13 ± 14.71	1526	51.27 ± 14.33	1.039 (1.029, 1.048)	<0.0001*
2. Recurrence regardless of vital status	414	54.23 ± 14.68	1372	51.49 ± 14.43	1.021 (1.013, 1.028)	<0.0001*
3. Death and/ or Recurrence	462	55.04 ± 14.76	1324	51.11 ± 14.31	1.025 (1.018, 1.032)	<0.0001*

[a] Among 1802 cases, only 1786 subjects were included:
- 2 Subjects were excluded due to missing first diagnosis and
- 14 subjects were excluded due to missing data for subsequent event assessment

[b] Hazard ratios, 95% confidence interval (CI) of hazard ratio, and p-values for were based on Wald test from the Cox proportional hazard analysis including age at first diagnosis (continuous) as the only variable in the model

Table 3.2 Relationship between Gender and Time to Event or Censored for Endpoints:
- **Death,**
- **Recurrence,**
- **Death and/or Recurrence**

Among Cases (Total N = 1786[a])

Endpoint	Event Rate: n / N (%)		Gender: Male vs. Female	
	Gender: Male	Gender: Female	Hazard Ratio (95& CI)[b]	p-Value[b]
1. Death regardless of recurrence status	178 / 1050 (16.95%)	82 / 736 (11.14%)	1.583 (1.218, 2.057)	0.0006*
2. Recurrence regardless of vital status	265 / 1050 (25.24%)	149 / 736 (20.24%)	1.303 (1.065, 1.593)	0.0100*
3. Death and/ or Recurrence	298 / 1050 (28.38%)	164 / 736 (22.28%)	1.334 (1.102, 1.615)	0.0031*

[a] Among 1802 cases, only 1786 subjects were included:
- 2 Subjects were excluded due to missing first diagnosis and
- 14 subjects were excluded due to missing data for subsequent event assessment

[b] Hazard ratios, 95% confidence interval (CI) of hazard ratio, and p-values for were based on Wald test from the Cox proportional hazard analysis including age at first diagnosis (continuous) as the only variable in the model

Table 3.3 Relationship between Stage and Time to Event or Censored for Endpoints:
- **Death,**
- **Recurrence,**
- **Death and/or Recurrence**

Among Cases (Total N = 1785[a])

Endpoint	Event Rate: n / N (%)		Stage II, III &IV vs. I & I/II	
	Stage: I & I/II	Stage: II, III, & IV	Hazard Ratio (95& CI)[b]	p-Value[b]
1. Death regardless of recurrence status	84 / 1095 (7.67%)	176 / 690 (25.51%)	4.404 (3.380, 5.739)	<.0001*
2. Recurrence regardless of vital status	155 / 1095 (14.16%)	259 / 690 (37.54%)	3.572 (2.921, 4.368)	<.0001*
3. Death and/ or Recurrence	174 / 1095 (15.89%)	288 / 690 (41.74%)	3.555 (2.939, 4.299)	<.0001*

[a] Among 1802 cases, only 1785 subjects were included:
- 2 Subjects were excluded due to missing first diagnosis and
- 14 subjects were excluded due to missing data for subsequent event assessment
- 1 subject with missing data for stage

[b] Hazard ratios, 95% confidence interval (CI) of hazard ratio, and p-values for were based on Wald test from the Cox proportional hazard analysis including age at first diagnosis (continuous) as the only variable in the model

Table 3.4 Relationship between Clark Level and Time to Event or Censored for Endpoints:
- **Death,**
- **Recurrence,**
- **Death and/or Recurrence**

Among Cases (Total N = 1543[a])

Endpoint	Event Rate: n / N (%)		Clark Level: 4 & 5 vs. 1 , 2, &3	
	Clark Level: 1, 2, &3	Clark Level: 4 & 5	Hazard Ratio (95& CI)[b]	p-Value[b]
1. Death regardless of recurrence status	51 / 820 (6.22%)	131 / 723 (18.12%)	3.104 (2.245, 4.291)	<.0001*
2. Recurrence regardless of vital status	67 / 820 (8.17%)	202 / 723 (27.94%)	3.971 (3.012, 5.237)	<.0001*
3. Death and/ or Recurrence	88 / 820 (10.73%)	222 / 723 (30.71%)	3.330 (2.601, 4.264)	<.0001*

[a] Among 1802 cases, only 1543 subjects were included:
- 2 Subjects were excluded due to missing first diagnosis and
- 14 subjects were excluded due to missing data for subsequent event assessment
- 243 subjects with missing data for Clark level

[b] Hazard ratios, 95% confidence interval (CI) of hazard ratio, and p-values for were based on Wald test from the Cox proportional hazard analysis including age at first diagnosis (continuous) as the only variable in the model

Table 4.1 Comparison of Time from First Diagnosis to Endpoint of Death (Regardless of Recurrent Status) among the 3 Allele Types for Each Gene Among Cases (Total N = 1543[a])- Unadjusted Analysis

	Endpoint = Death regardless of Recurrent Status Total Mortality Rate = 182 / 1543 (11.80%) Unadjusted Analysis				
	Event Rate: n / N (%) Copies of Ancestral Allele:			Ancestral Allele: Per 1 copy decrease	
Gene SNP	**2 copies**	**1 copy**	**0 copy**	**Hazard Ratio (95% CI)**	**P-value[b]**
IL-1					
rs1800587	85 / 733 (11.60%)	78 / 665 (11.73%)	19 / 145 (13.10%)	1.038 (0.834, 1.291)	0.7406
rs17561	85 / 733 (11.60%)	78 / 665 (11.73%)	19 / 145 (13.10%)	1.040 (0.836, 1.293)	0.7273
rs2071374	88 / 776 (11.34%)	84 / 663 (12.67%)	10 / 104 (9.62%)	1.042 (0.826, 1.315)	0.7287
rs16944	20 / 155 (12.90%)	89 / 701 (12.70%)	73 / 687 (10.63%)	0.891 (0.714, 1.113)	0.3103
rs1143627	20 / 155 (12.90%)	89 / 701 (12.70%)	73 / 687 (10.63%)	0.891 (0.714, 1.113)	0.3103
rs1071676	107 / 877 (12.20%)	66 / 564 (11.70%)	9 / 102 (8.82%)	0.943 (0.742, 1.200)	0.6351
rs1143634	107 / 880 (12.16%)	66 / 564 (11.70%)	9 / 99 (9.09%)	0.959 (0.753, 1.221)	0.7338
rs3136558	119 / 955 (12.46%)	55 / 504 (10.91)	8 / 84 (9.52%)	0.892 (0.690, 1.152)	0.3807
IL-6					
rs1800795	62 / 509 (12.18%)	84 / 753 (11.16%)	36 / 281 (12.81%)	1.005 (0.814, 1.240)	0.9659
rs1800797	63 / 530 (11.89%)	83 / 743 (11.17%)	36 / 270 (13.33%)	1.031 (0.836, 1.271)	0.7765

Table 4.1 (cont'd) Comparison of Time from First Diagnosis to Endpoint of Death (Regardless of Recurrent Status) among the 3 Allele Types for Each Gene Among Cases (Total N = 1543[a])- Unadjusted Analysis

	Endpoint = Death regardless of Recurrent Status Total Mortality Rate = 182 / 1543 (11.80%) Unadjusted Analysis				
	Event Rate: n / N (%) Copies of Ancestral Allele:			Ancestral Allele: Per 1 copy decrease	
Gene SNP	**2 copies**	**1 copy**	**0 copy**	**Hazard Ratio (95% CI)**	**P-value[b]**
IL-8					
rs4073	29 / 315 (9.21%)	100 / 758 (13.19%)	53 / 470 (11.28%)	1.090 (0.886, 1.341)	0.4150
rs2227307	53 / 483 (10.97%)	102 / 754 (13.53%)	27 / 306 (8.82%)	0.923 (0.750, 1.136)	0.4503
rs2227306	61 / 531 (11.49%)	95 / 738 (12.87%)	26 / 274 (9.49%)	0.921 (0.748, 1.134)	0.4371
IFN-G					
rs2430561	31 / 321 (9.66%)	83 / 752 (11.04%)	68 / 470 (14.47%)	1.227 (0.995, 1.514)	0.0559
rs1861494	19 / 140 (13.57%)	79 / 640 (12.34%)	84 / 763 (11.01%)	0.903 (0.725, 1.125)	0.3626
rs2069705	22 / 172 (12.79%)	86 / 698 (12.32%)	74 / 673 (11.00%)	0.914 (0.737, 1.133)	0.4125
TNF-A					
rs361525	169 / 1367 (12.36%)	13 / 171 (7.60%)	0 / 5 (0.00%)	0.593 (0.340, 1.036)	0.0666
rs1800629	135 / 1092 (12.36%)	42 / 408 (10.29%)	5 / 43 (11.63%)	0.901 (0.673, 1.205)	0.4805
rs1799964	5 / 82 (6.10%)	61 / 510 (11.96%)	116 / 951 (12.20%)	1.137 (0.882, 1.467)	0.3221

rs1800630	127 / 1090 (11.65%)	52 / 413 (12.59%)	3 / 40 (7.50%)	1.005 (0.758, 1.332)	0.9716

[a] Among 1802 cases, only 1543 subjects were included:
- 2 Subjects were excluded due to missing first diagnosis and
- 14 subjects were excluded due to missing data for subsequent event assessment
- 1 subject had missing data for both stage and Clark level; 242 subjects had missing data for Clark level

[b] Hazard ratios, 95% confidence interval (CI) of hazard ratio, and p-values were based on Wald test from Cox proportional
hazard analysis including copies of ancestral allele as the major predictor (continuous scale) .
hazard analysis including number of copies as the major predictor (continuous scale) .

Table 4.2 Comparison of Time from First Diagnosis to Endpoint of Death (Regardless of Recurrent Status) among the 3 Allele Types for Each SNP Among Cases (Total N = 1543[a]) - Final Model Built by Stepwise Selection Process

Endpoint = Death regardless of Recurrent Status
Total Mortality Rate = 182 / 1543 (11.80%)
Final Model: by Stepwise Selection process

	Event Rate: n / N (%) Copies of Ancestral Allele:			Ancestral Allele: Per 1 copy decrease		Covariates: Entered into the final model by step-wise selection process: Age at First DX. Per 1 Year Increase		Gender: Male vs. Female		Stage III & IV vs. I & I/II		Clark Level 4 & 5 vs. 1, 2, 3	
Gene SNP	**2 copies**	**1 copy**	**0 copy**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**
IL-1													
rs1800587	85 / 733 (11.60%)	78 / 665 (11.73%)	19 / 145 (13.10%)	0.948 (0.759, 1.185)	0.6405	1.031 (1.019, 1.042)	<.0001*	--------	-----	4.962 (3.369, 7.308)	<.0001*	1.388 (0.975, 1.975)	0.0688
rs17561	85 / 733 (11.60%)	78 / 665 (11.73%)	19 / 145 (13.10%)	0.947 (0.758, 1.183)	0.6297	1.031 (1.019, 1.042)	<.0001*	--------	-----	4.964 (3.371, 7.311)	<.0001*	1.388 (0.975, 1.975)	0.0688
rs2071374	88 / 776 (11.34%)	84 / 663 (12.67%)	10 / 104 (9.62%)	1.130 (0.894, 1.430)	0.3069	1.030 (1.019, 1.042)	<.0001*	--------	-----	4.970 (3.374, 7.321)	<.0001*	1.389 (0.976, 1.976)	0.0679
rs16944	20 / 155 (12.90%)	89 / 701 (12.70%)	73 / 687 (10.63%)	0.883 (0.712, 1.095)	0.2569	1.031 (1.019, 1.042)	<.0001*	--------	-----	4.947 (3.360, 7.285)	<.0001*	1.382 (0.972, 1.966)	0.0717
rs1143627	20 / 155 (12.90%)	89 / 701 (12.70%)	73 / 687 (10.63%)	0.883 (0.712, 1.095)	0.2569	1.031 (1.019, 1.042)	<.0001*	--------	-----	4.947 (3.360, 7.285)	<.0001*	1.382 (0.972, 1.966)	0.0717
rs1071676	107 / 877 (12.20%)	66 / 564 (11.70%)	9 / 102 (8.82%)	0.865 (0.673, 1.112)	0.2591	1.031 (1.019, 1.042)	<.0001*	--------	-----	4.963 (3.372, 7.305)	<.0001*	1.393 (0.980, 1.981)	0.0649
rs1143634	107 / 880 (12.16%)	66 / 564 (11.70%)	9 / 99 (9.09%)	0.870 (0.677, 1.119)	0.2792	1.031 (1.019, 1.042)	<.0001*	--------	-----	4.969 (3.376, 7.315)	<.0001*	1.392 (0.979, 1.980)	0.0654
rs3136558	119 / 955 (12.46%)	55 / 504 (10.91%)	8 / 84 (9.52%)	0.820 (0.632, 1.063)	0.1345	1.031 (1.020, 1.042)	<.0001*	--------	-----	4.973 (3.379, 7.321)	<.0001*	1.390 (0.978, 1.976)	0.0665

Table 4.2 (cont'd) **Comparison of Time from First Diagnosis to Endpoint of Death (Regardless of Recurrent Status) among the 3 Allele Types for Each SNP Among Cases (Total N = 1543[a])- Final Model Built by Stepwise Selection Process**

Endpoint = Death regardless of Recurrent Status
Total Mortality Rate = 182 / 1543 (11.80%)
Final Model: by Stepwise Selection process

						Covariates: Entered into the final model by step-wise selection process:							
	Event Rate: n / N (%) Copies of Ancestral Allele:			**Ancestral Allele: Per 1 copy decrease**		**Age at First DX. Per 1 Year Increase**		**Gender: Male vs. Female**		**Stage III & IV vs. I & I/II**		**Clark Level 4 & 5 vs. 1, 2, 3**	
Gene SNP	**2 copies**	**1 copy**	**0 copy**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**
IL-6													
rs1800795	62 / 509 (12.18%)	84 / 753 (11.16%)	36 / 281 (12.81%)	0.984 (0.795, 1.218)	0.8823	1.031 (1.019, 1.042)	<.0001*	--------	-----	4.952 (3.361, 7.295)	<.0001*	1.380 (0.970, 1.963)	0.0737
rs1800797	63 / 530 (11.89%)	83 / 743 (11.17%)	36 / 270 (13.33%)	1.017 (0.823, 1.256)	0.8779	1.031 (1.019, 1.042)	<.0001*	--------	-----	4.943 (3.355, 7.282)	<.0001*	1.381 (0.971, 1.965)	0.0729
IL-8													
rs4073	29 / 315 (9.21%)	100 / 758 (13.19%)	53 / 470 (11.28%)	1.048 (0.852, 1.289)	0.6584	1.031 (1.019, 1.042)	<.0001*	--------	-----	4.927 (3.344, 7.259)	<.0001*	1.386 (0.974, 1.973)	0.0698
rs2227307	53 / 483 (10.97%)	102 / 754 (13.53%)	27 / 306 (8.82%)	0.959 (0.779, 1.180)	0.6933	1.031 (1.019, 1.042)	<.0001*	--------	-----	4.930 (3.346, 7.263)	<.0001*	1.385 (0.973, 1.971)	0.0704
rs2227306	61 / 531 (11.49%)	95 / 738 (12.87%)	26 / 274 (9.49%)	0.942 (0.765, 1.160)	0.5746	1.031 (1.019, 1.042)	<.0001*	--------	-----	4.929 (3.346, 7.261)	<.0001*	1.386 (0.974, 1.972)	0.0697

Endpoint = Death regardless of Recurrent Status
Total Mortality Rate = 182 / 1543 (11.80%)
Final Model: by Stepwise Selection process

	Event Rate: n / N (%) Copies of Ancestral Allele:			Covariates: Entered into the final model by step-wise selection process:									
				Ancestral Allele: Per 1 copy decrease		Age at First DX. Per 1 Year Increase		Gender: Male vs. Female		Stage III & IV vs. I & I/II		Clark Level 4 & 5 vs. 1, 2, 3	
Gene SNP	2 copies	1 copy	0 copy	Hazard Ratio (95% CI)[b]	P-value[b]	Hazard Ratio (95% CI)[b]	P-value[b]	Hazard Ratio (95% CI)[b]	P-value[b]	Hazard Ratio (95% CI)[b]	P-value[b]	Hazard Ratio (95% CI)[b]	P-value[b]
IFN-G													
rs2430561	31 / 321 (9.66%)	83 / 752 (11.04%)	68 / 470 (14.47%)	1.184 (0.958, 1.463)	0.1175	1.031 (1.020, 1.043)	<.0001*	--------	-----	4.897 (3.328, 7.206)	<.0001*	1.391 (0.979, 1.978)	0.0655
rs1861494	19 / 140 (13.57%)	79 / 640 (12.34%)	84 / 763 (11.01%)	0.890 (0.712, 1.113)	0.3066	1.031 (1.019, 1.042)	<.0001*	--------	-----	4.929 (3.347, 7.259)	<.0001*	1.392 (0.978, 1.981)	0.0660
rs2069705	22 / 172 (12.79%)	86 / 698 (12.32%)	74 / 673 (11.00%)	0.906 (0.730, 1.124)	0.3694	1.031 (1.019, 1.042)	<.0001*	--------	-----	4.931 (3.348, 7.263)	<.0001*	1.390 (0.977, 1.978)	0.0672

Table 4.2 (cont'd) **Comparison of Time from First Diagnosis to Endpoint of Death (Regardless of Recurrent Status) among the 3 Allele Types for Each SNP Among Cases (Total N = 1543[a])- Final Model Built by Stepwise Selection Process**

Endpoint = Death regardless of Recurrent Status
Total Mortality Rate = 182 / 1543 (11.80%)
Final Model: by Stepwise Selection process

	Event Rate: n / N (%) Copies of Ancestral Allele:			Covariates: Entered into the final model by step-wise selection process: Ancestral Allele: Per 1 copy decrease		Age at First DX. Per 1 Year Increase		Gender: Male vs. Female		Stage III & IV vs. I & I/II		Clark Level 4 & 5 vs. 1, 2, 3	
Gene SNP	**2 copies**	**1 copy**	**0 copy**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**
TNF-A													
rs361525	169 / 1367 (12.36%)	13 / 171 (7.60%)	0 / 5 (0.00%)	0.614 (0.353, 1.066)	0.0832	1.030 (1.019, 1.042)	<.0001*	--------	-----	4.964 (3.370, 7.310)	<.0001*	1.373 (0.965, 1.955)	0.0782
rs1800629	135 / 1092 (12.36%)	42 / 408 (10.29%)	5 / 43 (11.63%)	0.954 (0.715, 1.274)	0.7510	1.031 (1.019, 1.042)	<.0001*	--------	-----	4.934 (3.348, 7.270)	<.0001*	1.382 (0.971, 1.966)	0.0723
rs1799964	5 / 82 (6.10%)	61 / 510 (11.96%)	116 / 951 (12.20%)	0.997 (0.775, 1.281)	0.9786	1.031 (1.019, 1.042)	<.0001*	--------	-----	4.948 (3.357, 7.294)	<.0001*	1.381 (0.970, 1.964)	0.0731
rs1800630	127 / 1090 (11.65%)	52 / 413 (12.59%)	3 / 40 (7.50%)	1.190 (0.898, 1.577)	0.2248	1.031 (1.020, 1.042)	<.0001*	--------	-----	5.046 (3.423, 7.439)	<.0001*	1.372 (0.965, 1.952)	0.0782

[a] Among 1802 cases, only 1543 subjects were included:
2 Subjects were excluded due to missing first diagnosis, 14 subjects were excluded due to missing data for subsequent event assessment, and 243 subjects were excluded due to missing data of age, gender, stage, and Clark level

[b] Hazard ratios, 95% confidence interval (CI) of hazard ratio, and p-values were based on the Wald test from a Cox proportional hazard model built by stepwise selection process by forcing the copies of ancestral allele as the major predictor (continuous scale) and the remaining covariates (age at diagnosis , gender, stage, and Clark level) were selected by stepwise process using significant level of 0.10 to be entered and kept in the final model.

Table 5.1 Comparison of Time from First Diagnosis to Endpoint of Recurrence (Regardless of Vital Status) among the 3 Allele Types for Each Gene Among Cases (Total N = 1543[a])- Unadjusted Analysis

	Endpoint = Recurrence regardless of Vital Status Total Event Rate = 269 / 1543 (17.43%) Unadjusted Analysis				
	Event Rate: n / N (%) Copies of Ancestral Allele:			Ancestral Allele: Per 1 copy decrease	
Gene SNP	**2 copies**	**1 copy**	**0 copy**	**Hazard Ratio (95% CI)**	**P-value[b]**
IL-1					
rs1800587:	135 / 733 (18.42%)	111 / 665 (16.69%)	23 / 145 (15.86%)	0.919 (0.763, 1.105)	0.3685
rs17561:	135 / 733 (18.42%)	111 / 665 (16.69%)	23 / 145 (15.86%)	0.920 (0.764, 1.107)	0.3766
rs2071374:	135 / 776 (17.40%)	122 / 663 (18.40%)	12 / 104 (11.54%)	0.958 (0.789, 1.163)	0.6620
rs16944:	26 / 155 (16.77%)	136 / 701 (19.40%)	107 / 687 (15.57%)	0.910 (0.759, 1.091)	0.3084
rs1143627:	26 / 155 (16.77%)	136 / 701 (19.40%)	107 / 687 (15.57%)	0.910 (0.759, 1.091)	0.3084
rs1071676:	152 / 877 (17.33%)	102 / 564 (18.09%)	15 / 102 (14.71%)	1.018 (0.840, 1.234)	0.8565
rs1143634:	152 / 880 (17.27%)	102 / 564 (18.09%)	15 / 99 (15.15%)	1.036 (0.853, 1.257)	0.7213
rs3136558:	174 / 955 (18.22%)	81 / 504 (16.07%)	14 / 84 (16.67%)	0.928 (0.755, 1.140)	0.4756
IL-6					
rs1800795	89 / 509 (17.49%)	136 / 753 (18.06%)	44 / 281 (15.66%)	0.958 (0.807, 1.138)	0.6280
rs1800797	95 / 530 (17.92%)	131 / 743 (17.63%)	43 / 270 (15.93%)	0.945 (0.796, 1.123)	0.5233

Table 5.1 (cont'd) Comparison of Time from First Diagnosis to Endpoint of Recurrence (Regardless of Vital Status) among the 3 Allele Types for Each Gene Among Cases (Total N = 1543[a])- Unadjusted Analysis

	Endpoint = Recurrence regardless of Vital Status Total Event Rate = 269 / 1543 (17.43%) Unadjusted Analysis				
	Event Rate: n / N (%) Copies of Ancestral Allele:			Ancestral Allele: Per 1 copy decrease	
Gene SNP	**2 copies**	**1 copy**	**0 copy**	**Hazard Ratio (95% CI)**	**P-value[b]**
IL-8					
rs4073	48 / 315 (15.24%)	142 / 758 (18.73%)	79 / 470 (16.81%)	1.040 (0.879, 1.231)	0.6439
rs2227307	80 / 483 (16.56%)	143 / 754 (18.97%)	46 / 306 (15.03%)	0.969 (0.819, 1.146)	0.7113
rs2227306	88 / 531 (16.57%)	141 / 738 (19.11%)	40 / 274 (14.60%)	0.962 (0.813, 1.140)	0.6571
IFN-G					
rs2430561	49 / 321 (15.26%)	126 / 752 (16.76%)	94 / 470 (20.00%)	1.142 (0.962, 1.356)	0.1280
rs1861494	27 / 140 (19.29%)	112 / 640 (17.50%)	130 / 763 (17.04%)	0.970 (0.808, 1.164)	0.7399
rs2069705	33 / 172 (19.19%)	122 / 698 (17.48%)	114 / 673 (16.94%)	0.966 (0.809, 1.154)	0.7045
TFN-A					
rs361525	246 / 1367 (18.00%)	22 / 171 (12.87%)	1 / 5 (20.00%)	0.727 (0.482, 1.096)	0.1282
rs1800629	191 / 1092 (17.49%)	69 / 408 (16.91%)	9 / 43 (20.93%)	1.017 (0.810, 1.277)	0.8862
rs1799964	13 / 82 (15.85%)	90 / 510 (17.65%)	166 / 951 (17.46%)	1.025 (0.837, 1.255)	0.8111
rs1800630	186 / 1090 (17.06%)	74 / 413 (17.92%)	9 / 40 (22.50%)	1.085 (0.866, 1.360)	0.4756

[a] Among 1802 cases, only 1543 subjects were included:
- 2 Subjects were excluded due to missing first diagnosis and
- 14 subjects were excluded due to missing data for subsequent event assessment
- 1 subject had missing data for both stage and Clark level; 242 subjects had missing data for Clark level

[b] Hazard ratios, 95% confidence interval (CI) of hazard ratio, and p-values were based on Wald test from Cox proportional hazard analysis including copies of ancestral allele as the major predictor (continuous scale) .

Table 5.2 **Comparison of Time from First Diagnosis to Endpoint of Recurrence (Regardless of Vital Status) among the 3 Allele Types for Each SNP Among Cases (Total N = 1543[a])- Final Model Built by Stepwise Selection Process**

Endpoint = Recurrence regardless of Vital Status
Total Event Rate = 269 / 1543 (17.43%)
Final Model: by Stepwise Selection process

						Covariates: Entered into the final model by step-wise selection process:							
	Event Rate: n / N (%) Copies of Ancestral Allele:			**Ancestral Allele: Per 1 copy decrease**		**Age at First DX. Per 1 Year Increase**		**Gender: Male vs. Female**		**Stage III & IV vs. I & I/II**		**Clark Level 4 & 5 vs. 1, 2, 3**	
Gene SNP	**2 copies**	**1 copy**	**0 copy**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**
IL-1													
rs1800587	135 / 733 (18.42%)	111 / 665 (16.69%)	23 / 145 (15.86%)	0.836 (0.694, 1.008)	0.0610	1.014 (1.005, 1.023)	0.0020*	--------	-----	4.160 (3.062, 5.652)	<.0001*	2.010 (1.483, 2.725)	<.0001*
rs17561	135 / 733 (18.42%)	111 / 665 (16.69%)	23 / 145 (15.86%)	0.835 (0.693, 1.007)	0.0588	1.014 (1.005, 1.023)	0.0020*	--------	-----	4.165 (3.065, 5.658)	<.0001*	2.009 (1.482, 2.723)	<.0001*
rs2071374	135 / 776 (17.40%)	122 / 663 (18.40%)	12 / 104 (11.54%)	1.061 (0.871, 1.291)	0.5567	1.014 (1.005, 1.023)	0.0020*	--------	-----	4.131 (3.038, 5.617)	<.0001*	1.990 (1.467, 2.699)	<.0001*
rs16944	26 / 155 (16.77%)	136 / 701 (19.40%)	107 / 687 (15.57%)	0.907 (0.760, 1.082)	0.2779	1.014 (1.005, 1.023)	0.0022*	--------	-----	4.120 (3.031, 5.600)	<.0001*	1.985 (1.464, 2.692)	<.0001*
rs1143627	26 / 155 (16.77%)	136 / 701 (19.40%)	107 / 687 (15.57%)	0.907 (0.760, 1.082)	0.2779	1.014 (1.005, 1.023)	0.0022*	--------	-----	4.120 (3.031, 5.600)	<.0001*	1.985 (1.464, 2.692)	<.0001*
rs1071676	152 / 877 (17.33%)	102 / 564 (18.09%)	15 / 102 (14.71%)	0.959 (0.784, 1.171)	0.6792	1.014 (1.005, 1.023)	0.0019*	--------	-----	4.119 (3.030, 5.599)	<.0001*	1.988 (1.466, 2.695)	<.0001*

rs1143634	152 / 880 (17.27%)	102 / 564 (18.09%)	15 / 99 (15.15%)	0.966 (0.790, 1.181)	0.7341	1.014 (1.005, 1.023)	0.0020*	--------	-----	4.120 (3.031, 5.601)	<.0001*	1.987 (1.465, 2.694)	<.0001*
rs3136558	174 / 955 (18.22%)	81 / 504 (16.07%)	14 / 84 (16.67%)	0.863 (0.699, 1.065)	0.1686	1.014 (1.005, 1.023)	0.0018*	--------	-----	4.142 (3.047, 5.631)	<.0001*	1.986 (1.465, 2.693)	<.0001*

Table 5.2(cont'd) **Comparison of Time from First Diagnosis to Endpoint of Recurrence (Regardless of Vital Status) among the 3 Allele Types for Each SNP Among Cases (Total N = 1543[a])- Final Model Built by Stepwise Selection Process**

Endpoint = Recurrence regardless of Vital Status
Total Event Rate = 269 / 1543 (17.43%)
Final Model: by Stepwise Selection process

						Covariates: Entered into the final model by step-wise selection process:							
	Event Rate: n / N (%) Copies of Ancestral Allele:			**Ancestral Allele: Per 1 copy decrease**		**Age at First DX. Per 1 Year Increase**		**Gender: Male vs. Female**		**Stage III & IV vs. I & I/II**		**Clark Level 4 & 5 vs. 1, 2, 3**	
Gene SNP	**2 copies**	**1 copy**	**0 copy**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**
IL-6													
rs1800795	89 / 509 (17.49%)	136 / 753 (18.06%)	44 / 281 (15.66%)	0.938 (0.790, 1.113)	0.4629	1.014 (1.005, 1.023)	0.0019*	--------	-----	4.131 (3.039, 5.615)	<.0001*	1.980 (1.460, 2.685)	<.0001*
rs1800797	95 / 530 (17.92%)	131 / 743 (17.63%)	43 / 270 (15.93%)	0.931 (0.785, 1.105)	0.4123	1.014 (1.005, 1.023)	0.0019*	--------	-----	4.128 (3.037, 5.610)	<.0001*	1.981 (1.461, 2.686)	<.0001*
IL-8													
rs4073	48 / 315 (15.24%)	142 / 758 (18.73%)	79 / 470 (16.81%)	1.021 (0.863, 1.207)	0.8091	1.014 (1.005, 1.023)	0.0021*	--------	-----	4.110 (3.024, 5.588)	<.0001*	1.988 (1.466, 2.696)	<.0001*
rs2227307	80 / 483 (16.56%)	143 / 754 (18.97%)	46 / 306 (15.03%)	0.984 (0.832, 1.165)	0.8545	1.014 (1.005, 1.023)	0.0021*	--------	-----	4.112 (3.025, 5.590)	<.0001*	1.987 (1.465, 2.695)	<.0001*
rs2227306	88 / 531 (16.57%)	141 / 738 (19.11%)	40 / 274 (14.60%)	0.963 (0.813, 1.141)	0.6618	1.014 (1.005, 1.023)	0.0021*	--------	-----	4.110 (3.024, 5.587)	<.0001*	1.990 (1.467, 2.699)	<.0001*

Table 5.2(cont'd) **Comparison of Time from First Diagnosis to Endpoint of Recurrence (Regardless of Vital Status) among the 3 Allele Types for Each SNP Among Cases (Total N = 1543[a])- Final Model Built by Stepwise Selection Process**

Endpoint = Recurrence regardless of Vital Status
Total Event Rate = 269 / 1543 (17.43%)
Final Model: by Stepwise Selection process

						Covariates: Entered into the final model by step-wise selection process:							
	Event Rate: n / N (%) Copies of Ancestral Allele:			**Ancestral Allele: Per 1 copy decrease**		**Age at First DX. Per 1 Year Increase**		**Gender: Male vs. Female**		**Stage III & IV vs. I & I/II**		**Clark Level 4 & 5 vs. 1, 2, 3**	
Gene SNP	**2 copies**	**1 copy**	**0 copy**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**
IFN-G													
rs2430561	49 / 321 (15.26%)	126 / 752 (16.76%)	94 / 470 (20.00%)	1.116 (0.940, 1.324)	0.2088	1.014 (1.005, 1.023)	0.0019*	--------	-----	4.107 (3.024, 5.579)	<.0001*	1.990 (1.468, 2.697)	<.0001*
rs1861494	27 / 140 (19.29%)	112 / 640 (17.50%)	130 / 763 (17.04%)	0.943 (0.784, 1.134)	0.5327	1.014 (1.005, 1.023)	0.0020*	--------	-----	4.117 (3.029, 5.595)	<.0001*	1.990 (1.468, 2.699)	<.0001*
rs2069705	33 / 172 (19.19%)	122 / 698 (17.48%)	114 / 673 (16.94%)	0.944 (0.789, 1.128)	0.5241	1.014 (1.005, 1.023)	0.0021*	--------	-----	4.117 (3.029, 5.595)	<.0001*	1.989 (1.467, 2.698)	<.0001*
TNF-A													
rs361525	246 / 1367 (18.00%)	22 / 171 (12.87%)	1 / 5 (20.00%)	0.721 (0.479, 1.086)	0.1172	1.014 (1.005, 1.022)	0.0025*	--------	-----	4.120 (3.030, 5.600)	<.0001*	1.989 (1.467, 2.699)	<.0001*
rs1800629	191 / 1092 (17.49%)	69 / 408 (16.91%)	9 / 43 (20.93%)	1.098 (0.880, 1.370)	0.4070	1.014 (1.005, 1.023)	0.0022*	--------	-----	4.140 (3.045, 5.629)	<.0001*	1.986 (1.465, 2.693)	<.0001*
rs1799964	13 / 82 (15.85%)	90 / 510 (17.65%)	166 / 951 (17.46%)	0.944 (0.773, 1.153)	0.5737	1.014 (1.005, 1.023)	0.0018*	--------	-----	4.137 (3.042, 5.625)	<.0001*	1.980 (1.460, 2.685)	<.0001*
rs1800630	186 / 1090 (17.06%)	74 / 413 (17.92%)	9 / 40 (22.50%)	1.220 (0.974, 1.527)	0.0832	1.014 (1.005, 1.023)	0.0014*	--------	-----	4.200 (3.088, 5.713)	<.0001*	1.967 (1.451, 2.667)	<.0001*

[a] Same as table 4.2
[b] Same as table 4.2

Table 6.1 Comparison of Time from First Diagnosis to the Composite Endpoint of Death and/ or Recurrence among the 3 Allele Types for Each Gene Among Cases (Total N = 1543[a])- Unadjusted Analysis

	Composite Endpoint = Death and/or Recurrent Total Event Rate = 310 / 1543 (20.09%) Unadjusted Analysis				
	Event Rate: n / N (%) Copies of Ancestral Allele:			Ancestral Allele: Per 1 copy decrease	
Gene SNP	**2 copies**	**1 copy**	**0 copy**	**Hazard Ratio (95% CI)**	**P-value[b]**
IL-1					
rs1800587:	158 / 733 (21.56%)	126 / 665 (18.95%)	26 / 145 (17.93%)	0.893 (0.751, 1.062)	0.1996
rs17561:	158 / 733 (21.56%)	126 / 665 (18.95%)	26 / 145 (17.93%)	0.894 (0.752, 1.063)	0.2060
rs2071374:	149 / 776 (19.20%)	145 / 663 (21.87%)	16 / 104 (15.38%)	1.035 (0.866, 1.237)	0.7039
rs16944:	28 / 155 (18.06%)	157 / 701 (22.40%)	125 / 687 (18.20%)	0.934 (0.788 1.107)	0.4309
rs1143627:	28 / 155 (18.06%)	157 / 701 (22.40%)	125 / 687 (18.20%)	0.934 (0.788 1.107)	0.4309
rs1071676:	179 / 877 (20.41%)	115 / 564 (20.39%)	16 / 102 (15.69%)	0.969 (0.809, 1.162)	0.7376
rs1143634:	179 / 880 (20.34%)	115 / 564 (20.39%)	16 / 99 (16.16%)	0.985 (0.821, 1.183)	0.8747
rs3136558:	199 / 955 (20.84%)	96 / 504 (19.05%)	15 / 84 (17.86%)	0.928 (0.766, 1.124)	0.4442
IL-6					
rs1800795	103 / 509 (20.24%)	154 / 753 (20.45%)	53 / 281 (18.86%)	0.972 (0.828, 1.141)	0.7263
rs1800797	109 / 530 (20.57%)	149 / 743 (20.05%)	52 / 270 (19.26%)	0.965 (0.822, 1.133)	0.6657

Table 6.1 (cont'd) Comparison of Time from First Diagnosis to the Composite Endpoint of Death and/ or Recurrence among the 3 Allele Types for Each Gene Among Cases (Total N = 1543[a])- Unadjusted Analysis

	Composite Endpoint = Death and/or Recurrent Total Event Rate = 310 / 1543 (20.09%) Unadjusted Analysis				
	Event Rate: n / N (%) Copies of Ancestral Allele:			Ancestral Allele: Per 1 copy decrease	
Gene SNP	**2 copies**	**1 copy**	**0 copy**	**Hazard Ratio (95% CI)**	**P-value[b]**
IL-8					
rs4073	51 / 315 (16.19%)	167 / 758 (22.03%)	92 / 470 (19.57%)	1.075 (0.919, 1.258)	0.3662
rs2227307	93 / 483 (19.25%)	168 / 754 (22.28%)	49 / 306 (16.01%)	0.940 (0.803, 1.100)	0.4395
rs2227306	106 / 531 (19.96%)	161 / 738 (21.82%)	43 / 274 (15.69%)	0.915 (0.781, 1.072)	0.2700
IFN-G					
rs2430561	55 / 321 (17.13%)	147 / 752 (19.55%)	108 / 470 (22.98%)	1.152 (0.981, 1.352)	0.0843
rs1861494	29 / 140 (20.71%)	130 / 640 (20.31%)	151 / 763 (19.79%)	0.996 (0.840, 1.182)	0.9643
rs2069705	35 / 172 (20.35%)	143 / 698 (20.49%)	132 / 673 (19.61%)	0.992 (0.840, 1.172)	0.9257
TNF-A					
rs361525	284 / 1367 (20.78%)	25 / 171 (14.62%)	1 / 5 (20.00%)	0.703 (0.477, 1.036)	0.0751
rs1800629	224 / 1092 (20.51%)	77 / 408 (18.87%)	9 / 43 (20.93%)	0.953 (0.767, 1.184)	0.6662
rs1799964	13 / 82 (15.85%)	105 / 510 (20.59%)	192 / 951 (20.19%)	1.054 (0.872, 1.274)	0.5884
rs1800630	215 / 1090 (19.72%)	86 / 413 (20.82%)	9 / 40 (22.50%)	1.059 (0.857, 1.309)	0.5929

- 2 Subjects were excluded due to missing first diagnosis and
- 14 subjects were excluded due to missing data for subsequent event assessment
- 1 subject had missing data for both stage and Clark level; 242 subjects had missing data for Clark level

[b] Hazard ratios, 95% confidence interval (CI) of hazard ratio, and p-values were based on Wald test from Cox proportional hazard analysis including copies of ancestral allele as the major predictor (continuous scale) .

Table 6.2 Comparison of Time from First Diagnosis to the Composite Endpoint of Death and / or Recurrent among the 3 Allele Types for Each SNP Among Cases (Total N = 1543[a])- Final Model Built by Stepwise Selection Process

Endpoint = Death and/or Recurrent
Total EventRate = 310 / 1543 (20.09%)
Final Model: by Stepwise Selection process

	Event Rate: n / N (%) Copies of Ancestral Allele:			Ancestral Allele: Per 1 copy decrease		Covariates: Entered into the final model by step-wise selection process: Age at First DX. Per 1 Year Increase		Gender: Male vs. Female		Stage III & IV vs. I & I/II		Clark Level 4 & 5 vs. 1, 2, 3	
Gene SNP	**2 copies**	**1 copy**	**0 copy**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**
IL-1													
rs1800587	158 / 733 (21.56%)	126 / 665 (18.95%)	26 / 145 (17.93%)	0.823 (0.691, 0.979)	0.0283 *	1.020 (1.012, 1.028)	<.0001 *	--------	-----	3.934 (2.973, 5.206)	<.0001 *	1.683 (1.280, 2.212)	0.0002 *
rs17561	158 / 733 (21.56%)	126 / 665 (18.95%)	26 / 145 (17.93%)	0.821 (0.690, 0.978)	0.0270 *	1.020 (1.012, 1.028)	<.0001 *	--------	-----	3.939 (2.977, 5.213)	<.0001 *	1.681 (1.279, 2.210)	0.0002 *
rs2071374	149 / 776 (19.20%)	145 / 663 (21.87%)	16 / 104 (15.38%)	1.139 (0.951, 1.364)	0.1577	1.020 (1.011, 1.028)	<.0001 *	--------	-----	3.927 (2.964, 5.202)	<.0001 *	1.670 (1.270, 2.196)	0.0002 *
rs16944	28 / 155 (18.06%)	157 / 701 (22.40%)	125 / 687 (18.20%)	0.934 (0.792 1.103)	0.4225	1.020 (1.011, 1.028)	<.0001 *	--------	-----	3.893 (2.940, 5.155)	<.0001 *	1.660 (1.262, 2.183)	0.0003 *
rs1143627	28 / 155 (18.06%)	157 / 701 (22.40%)	125 / 687 (18.20%)	0.934 (0.792 1.103)	0.4225	1.020 (1.011, 1.028)	<.0001 *	--------	-----	3.893 (2.940, 5.155)	<.0001 *	1.660 (1.262, 2.183)	0.0003 *

rs1071676	179 / 877 (20.41%)	115 / 564 (20.39%)	16 / 102 (15.69%)	0.914 (0.757, 1.104)	0.3505	1.020 (1.012, 1.029)	<.0001 *	--------	-----	3.898 (2.944, 5.161)	<.0001 *	1.665 (1.266, 2.189)	0.0003 *
rs1143634	179 / 880 (20.34%)	115 / 564 (20.39%)	16 / 99 (16.16%)	0.921 (0.762, 1.113)	0.3930	1.020 (1.012, 1.029)	<.0001 *	--------	-----	3.901 (2.946, 5.165)	<.0001 *	1.664 (1.266, 2.188)	0.0003 *
rs3136558	199 / 955 (20.84%)	96 / 504 (19.05%)	15 / 84 (17.86%)	0.868 (0.714, 1.054)	0.1533	1.020 (1.012, 1.029)	<.0001 *	--------	-----	3.912 (2.955, 5.180)	<.0001 *	1.662 (1.264, 2.185)	0.0003 *

Table 6.2(cont'd) **Comparison of Time from First Diagnosis to the Composite Endpoint of Death and / or Recurrent among the 3 Allele Types for Each SNP Among Cases (Total N = 1543[a])- Final Model Built by Stepwise Selection Process**

Endpoint = Death and/or Recurrent
Total Event Rate = 310 / 1543 (20.09%)
Final Model: by Stepwise Selection process

Covariates: Entered into the final model by step-wise selection process:

Gene SNP	Event Rate: n / N (%) Copies of Ancestral Allele: 2 copies	1 copy	0 copy	Ancestral Allele: Per 1 copy decrease Hazard Ratio (95% CI)[b]	P-value[b]	Age at First DX. Per 1 Year Increase Hazard Ratio (95% CI)[b]	P-value[b]	Gender: Male vs. Female Hazard Ratio (95% CI)[b]	P-value[b]	Stage III & IV vs. I & I/II Hazard Ratio (95% CI)[b]	P-value[b]	Clark Level 4 & 5 vs. 1, 2, 3 Hazard Ratio (95% CI)[b]	P-value[b]
IL-6													
rs1800795	103 / 509 (20.24%)	154 / 753 (20.45%)	53 / 281 (18.86%)	0.949 (0.808, 1.114)	0.5187	1.020 (1.012, 1.029)	<.0001 *	--------	-----	3.903 (2.947, 5.167)	<.0001 *	1.658 (1.261, 2.180)	0.0003 *
rs1800797	109 / 530 (20.57%)	149 / 743 (20.05%)	52 / 270 (19.26%)	0.948 (0.807, 1.112)	0.5096	1.020 (1.012, 1.029)	<.0001 *	--------	-----	3.899 (2.945, 5.162)	<.0001 *	1.659 (1.261, 2.181)	0.0003 *
IL-8													
rs4073	51 / 315 (16.19%)	167 / 758 (22.03%)	92 / 470 (19.57%)	1.051 (0.898, 1.229)	0.5348	1.020 (1.012, 1.028)	<.0001 *	--------	-----	3.880 (2.931, 5.138)	<.0001 *	1.666 (1.267, 2.191)	0.0003 *
rs2227307	93 / 483 (19.25%)	168 / 754 (22.28%)	49 / 306 (16.01%)	0.958 (0.819, 1.121)	0.5960	1.020 (1.012, 1.028)	<.0001 *	--------	-----	3.883 (2.932, 5.141)	<.0001 *	1.665 (1.266, 2.189)	0.0003 *
rs2227306	106 / 531 (19.96%)	161 / 738 (21.82%)	43 / 274 (15.69%)	0.920 (0.785, 1.079)	0.3042	1.020 (1.011, 1.028)	<.0001 *	--------	-----	3.881 (2.932, 5.139)	<.0001 *	1.669 (1.269, 2.195)	0.0002 *

IFN-G													
rs2430561	55 / 321 (17.13%)	147 / 752 (19.55%)	108 / 470 (22.98%)	1.127 (0.960, 1.323)	0.1428	1.020 (1.012, 1.029)	<.0001 *	--------	-----	3.882 (2.934, 5.136)	<.0001 *	1.666 (1.268, 2.189)	0.0003 *
rs1861494	29 / 140 (20.71%)	130 / 640 (20.31%)	151 / 763 (19.79%)	0.970 (0.816, 1.153)	0.7280	1.020 (1.012, 1.028)	<.0001 *	--------	-----	3.890 (2.938, 5.150)	<.0001 *	1.664 (1.265, 2.189)	0.0003 *
rs2069705	35 / 172 (20.35%)	143 / 698 (20.49%)	132 / 673 (19.61%)	0.969 (0.820, 1.146)	0.7163	1.020 (1.012, 1.028)	<.0001 *	--------	-----	3.890 (2.938, 5.150)	<.0001 *	1.664 (1.265, 2.188)	0.0003 *

Table 6.2(cont'd) **Comparison of Time from First Diagnosis to the Composite Endpoint of Death and / or Recurrent among the 3 Allele Types for Each SNP Among Cases (Total N = 1543[a])- Final Model Built by Stepwise Selection Process**

Endpoint = Death and/or Recurrent
Total Event Rate = 310 / 1543 (20.09%)
Final Model: by Stepwise Selection process

	Event Rate: n / N (%) Copies of Ancestral Allele:			Ancestral Allele: Per 1 copy decrease		Covariates: Entered into the final model by step-wise selection process: Age at First DX. Per 1 Year Increase		Gender: Male vs. Female		Stage III & IV vs. I & I/II		Clark Level 4 & 5 vs. 1, 2, 3	
Gene SNP	**2 copies**	**1 copy**	**0 copy**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**	**Hazard Ratio (95% CI)[b]**	**P-value[b]**
TNF-A													
rs361525	284 / 1367 (20.78%)	25 / 171 (14.62%)	1 / 5 (20.00%)	0.707 (0.481, 1.040)	0.0784	1.020 (1.011, 1.028)	<.0001 *	--------	-----	3.898 (2.944, 5.162)	<.0001 *	1.661 (1.263, 2.186)	0.0003 *
rs1800629	224 / 1092 (20.51%)	77 / 408 (18.87%)	9 / 43 (20.93%)	1.023 (0.827, 1.265)	0.8354	1.020 (1.012, 1.028)	<.0001 *	--------	-----	3.896 (2.941, 5.161)	<.0001 *	1.661 (1.263, 2.185)	0.0003 *
rs1799964	13 / 82 (15.85%)	105 / 510 (20.59%)	192 / 951 (20.19%)	0.958 (0.795, 1.156)	0.6560	1.020 (1.012, 1.029)	<.0001 *	--------	-----	3.905 (2.948, 5.174)	<.0001 *	1.659 (1.262, 2.182)	0.0003 *
rs1800630	215 / 1090 (19.72%)	86 / 413 (20.82%)	9 / 40 (22.50%)	1.206 (0.976, 1.491)	0.0825	1.020 (1.012, 1.029)	<.0001 *	--------	-----	3.969 (2.996, 5.259)	<.0001 *	1.650 (1.255, 2.169)	0.0003 *

[a] Among 1802 cases, only 1543 subjects were included:
2 Subjects were excluded due to missing first diagnosis, 14 subjects were excluded due to missing data for subsequent event assessment, and 243 subjects were excluded due to missing data of age, gender, stage, and Clark level

[b] Hazard ratios, 95% confidence interval (CI) of hazard ratio, and p-values were based on the Wald test from a Cox proportional hazard model built by stepwise selection process by forcing the copies of ancestral allele as the major predictor (continuous scale) and the remaining covariates (age at diagnosis , gender, stage, and Clark level) were selected by stepwise process using significant level of 0.10 to be entered and kept in the final model

Table 7.1 Interaction of IL-1 SNPs rs1800587 and rs7561 for Outcome of Death

	Endpoint = Recurrence regardless of Vital Status Total Event Rate = 269 / 1543[a] (17.43%) Event Rate: n / N (%) Copies of Ancestral Allele:									Ancestral Allele on Individual SNP: Per 1 copy decrease		Interaction between 2 SNPs
Individual SNP:	**2 copies**			**1 copy**			**0 copies**			**Hazard Ratio (95% CI)[b]**	**P-value[b]**	**P-value[c]**
IL-1 rs1800587	135 / 733 (18.42%)			111 / 665 (16.69%)			23 / 145 (15.86%)			0.836 (0.694, 1.008)	0.0610	
IL-1 rs17561	135 / 733 (18.42%)			111 / 665 (16.69%)			23 / 145 (15.86%)			0.835 (0.693, 1.007)	0.0588	
Combination of 2 SNPs:												
IL-1 rs1800587 And	**2 copies**			**1 copy**			**0 copies**					
IL-1 rs7561	**2 copies**	**1 copy**	**0 copy**	**2 copies**	**1 copy**	**0 copy**	**2 copies**	**1 copy**	**0 copy**			
	135/732 (18.44%)	0 / 1 (0%)	0 / 0	0 / 0	111 / 664 (16.72%)	0 / 1 (0%)	0 / 0	0 / 0	23 / 145 (15.86%)			0.9090

[a] Among 1802 cases, only 1543 subjects were included:
2 Subjects were excluded due to missing first diagnosis,14 subjects were excluded due to missing data for subsequent event assessment, and 243 subjects were excluded due to missing data of age, gender, stage, and Clark level

[b] Hazard ratios, 95% confidence interval (CI) of hazard ratio, and p-values were based on the Wald test from a Cox proportional hazard model built by stepwise selection process forcing the copies of ancestral allele as the major predictor (continuous scale) and the remaining covariates (age at diagnosis , gender, stage, and Clark level) were selected by stepwise process using significant level of 0.10 to be entered and kept in the final model.

[c] Interaction between two SNPs was based on the Wald test of the interaction term between two SNPs from a Cox proportional hazard model built by stepwise selection process forcing the copies of ancestral allele on two alleles as two major predictor (continuous scale) along with the corresponding interaction term and the remaining covariates (age at diagnosis , gender, stage, and Clark level) were selected by stepwise process using significant level of 0.10 to be entered and kept in the final model.

Table 7.2 Interaction of IL-1 SNPs rs1800587 and rs7561 for Composite Outcome

	Endpoint = Death and/or Recurrent Total Event Rate = 310 / 1543 (20.09%) Final Model: by Stepwise Selection process Event Rate: n / N (%) Copies of Ancestral Allele:									Ancestral Allele on Individual SNP: Per 1 copy decrease		Interaction between 2 SNPs
Individual SNP:	**2 copies**			**1 copy**			**0 copies**			**Hazard Ratio (95% CI)[b]**	**P-value[b]**	**P-value[c]**
IL-1 rs1800587	158 / 733 (21.56%)			126 / 665 (18.95%)			26 / 145 (17.93%)			0.823 (0.691, 0.979)	0.0283*	
IL-1 rs17561	158 / 733 (21.56%)			126 / 665 (18.95%)			26 / 145 (17.93%)			0.821 (0.690, 0.978)	0.0270*	
Combination of 2 SNPs:												
IL-1 rs1800587 and	**2 copies**			**1 copy**			**0 copies**					
IL-1 rs7561	**2 copies**	**1 copy**	**0 copy**	**2 copies**	**1 copy**	**0 copy**	**2 copies**	**1 copy**	**0 copy**			
	158/732 (21.58%)	0 / 1 (0%)	0 / 0	0 / 1 (0%)	126 / 664 (18.98%)	0 / 0	0 / 0	0 / 0	26 / 145 (17.93%)			0.9089

[a] Among 1802 cases, only 1543 subjects were included:
2 Subjects were excluded due to missing first diagnosis, 14 subjects were excluded due to missing data for subsequent event assessment, and 243 subjects were excluded due to missing data of age, gender, stage, and Clark level

[b] Hazard ratios, 95% confidence interval (CI) of hazard ratio, and p-values were based on the Wald test from a Cox proportional hazard model built by stepwise selection process forcing the copies of ancestral allele as the major predictor (continuous scale) and the remaining covariates (age at diagnosis , gender, stage, and Clark level) were selected by stepwise process using significant level of 0.10 to be entered and kept in the final model.

[c] Interaction between two SNPs was based on the Wald test of the interaction term between two SNPs from a Cox proportional hazard model built by stepwise selection process forcing the copies of ancestral allele on two alleles as two major predictor (continuous scale) along with the corresponding interaction term and the remaining covariates (age at diagnosis , gender, stage, and Clark level) were selected by stepwise process using significant level of 0.10 to be entered and kept in the final model.

Table 7.3 Interaction of TNF-A SNPs rs1800360 and rs361525 for Outcome of Death

Endpoint = Death and/or Recurrent Total Event Rate = 310 / 1543 (20.09%) Final Model: by Stepwise Selection process Event Rate: n / N (%) Copies of Ancestral Allele:										Ancestral Allele on Individual SNP: Per 1 copy decrease		Interaction between 2 SNPs
Individual SNP:	**2 copies**			**1 copy**			**0 copies**			**Hazard Ratio (95% CI)[b]**	**P-value[b]**	**P-value[c]**
TNF-A rs361525	284 / 1367 (20.78%)			25 / 171 (14.62%)			1 / 5 (20.00%)			0.707 (0.481, 1.040)	0.0784	
TNF-A rs1800360	215 / 1090 (19.72%)			86 / 413 (20.82%)			9 / 40 (22.50%)			1.206 (0.976, 1.491)	0.0825	
Combination of 2 SNPs:												
TNF-A rs361525 And	**2 copies**			**1 copy**			**0 copies**					
TNF-A rs1800360	**2 copies**	**1 copy**	**0 copy**	**2 copies**	**1 copy**	**0 copy**	**2 copies**	**1 copy**	**0 copy**			
	192/951 (20.19%)	83 / 376 (22.07%)	9 / 40 (22.50%)	22/134 (16.42%)	3 / 37 (8.11%)	0 / 0	1 / 5 (20%)	0 / 0	0 / 0			0.0652

[a] Among 1802 cases, only 1543 subjects were included:
2 Subjects were excluded due to missing first diagnosis, 14 subjects were excluded due to missing data for subsequent event assessment, and 243 subjects were excluded due to missing data of age, gender, stage, and Clark level

[b] Hazard ratios, 95% confidence interval (CI) of hazard ratio, and p-values were based on the Wald test from a Cox proportional hazard model built by stepwise selection process forcing the copies of ancestral allele as the major predictor (continuous scale) and the remaining covariates (age at diagnosis , gender, stage, and Clark level) were selected by stepwise process using significant level of 0.10 to be entered and kept in the final model.

[c] Interaction between two SNPs was based on the Wald test of the interaction term between two SNPs from a Cox proportional hazard model built by stepwise selection process forcing the copies of ancestral allele on two alleles as two major predictor (continuous scale) along with the corresponding interaction term and the remaining covariates (age at diagnosis , gender, stage, and Clark level) were selected by stepwise process using significant level of 0.10 to be entered and kept in the final model.

French Validation Results

Table 8 French Validation - Dr. Florence Demenais[a] - Significant Findings

gene	SNP	Study Ednpoint= Death	
		Hazard ratio [95% CI][b]	p-value[b]
IL-1B	rs3136558	0.685 [0.011, 42.803]	0.0719
IL-6	rs1800797	1.495 [1.116, 2.002]	0.007*
IL-6	rs1800795	1.501 [1.127, 1.998],	0.005*

[a] This validation was performed by the group of Dr. Florence Demenais, Directrice d'Unité : DR1 Inserm, of l'Université Paris Diderot - Paris 7, l'Institut Universitaire d'Hematologie. They used independent French data.
[b] Hazard ratios and p-values were calculated for per one ancestral allele copy decrease for individual SNPs based on a multivariate final model selected via stepwise Cox proportional hazard analysis from a set of covariates: age, gender, ethnicity , tumor stage, Clark level, and Breslow index.

Table 9 Pathway Analysis – Genes of the IL1 Pathway Significant for Outcome of Recurrence, adjusted for Age of Diagnosis, Gender, Clark Level, Tumor Stage, and Breslow Thickness

Gene	Endpoint = Recurrence
	P-value
IL-1	0.035*
ECSIT	0.02*
c-JUN	0.008*
TAK1	0.074*

Table 10 **Pathway Analysis – All Genes of the IL1 Pathway for Outcomes of Death and Recurrence, unadjusted and adjusted for Age of Diagnosis, Gender, Clark Level, Tumor Stage, and Breslow Thickness, including number of SNPs**

	Endpoint = Death Unadjusted	Endpoint = Death Adjusted	Endpoint = Recurrence Unadjusted	Endpoint = Recurrence Adjusted	Number of SNPs included for each Gene
Gene	**P-value**	**P-value**	**P-value**	**P-value**	
IL-1	0.315	0.122	0.115	0.035*	539
IL-6	0.122	0.507	0.176	0.467	719
IL-8	0.202	0.386	0.338	0.522	377
IFN-G	0.619	0.972	0.425	0.893	657
TNF-A	0.785	0.608	0.933	0.824	499
c-JUN	0.35	0.18	0.279	0.008*	193
ECSIT	0.632	0.188	0.158	0.02*	193
IkBa	0.448	0.263	0.76	0.536	193
IKKa	0.448	0.263	0.76	0.536	193
IKKb	0.042	0.475	0.072	0.611	193
IL1R1	0.962	0.889	0.35	0.803	193
IL1RA	0.63	0.848	0.68	0.784	193
IL1RAcP	0.854	0.644	0.947	0.737	193
IRAK4	0.533	0.357	0.385	0.145	193
JNK	0.337	0.918	0.231	0.955	193
MEK3	0.396	0.73	0.639	0.865	193
MEK6	0.617	0.47	0.727	0.579	193
MEKK1	0.693	0.862	0.719	0.889	193
MYD88	0.906	0.424	0.701	0.584	193

Gene	Endpoint = Death Unadjusted P-value	Endpoint = Death Adjusted P-value	Endpoint = Recurrence Unadjusted P-value	Endpoint = Recurrence P-value	Number of SNPs included for each Gene
NF-kB	0.246	0.548	0.272	0.489	193
NIK	0.644	0.687	0.639	0.218	193
P38	0.643	0.621	0.803	0.909	193
RKIP	0.753	0.87	0.968	0.973	193
TAB1	0.367	0.633	0.263	0.526	193
TAK1	0.281	0.123	0.459	0.074*	193
TOLLIP	0.404	0.635	0.534	0.7	193
TRAF6	0.187	0.553	0.275	0.632	193

Figure 3. Q-Q Plot of Adjusted Probabilities of Genes for Death Outcome

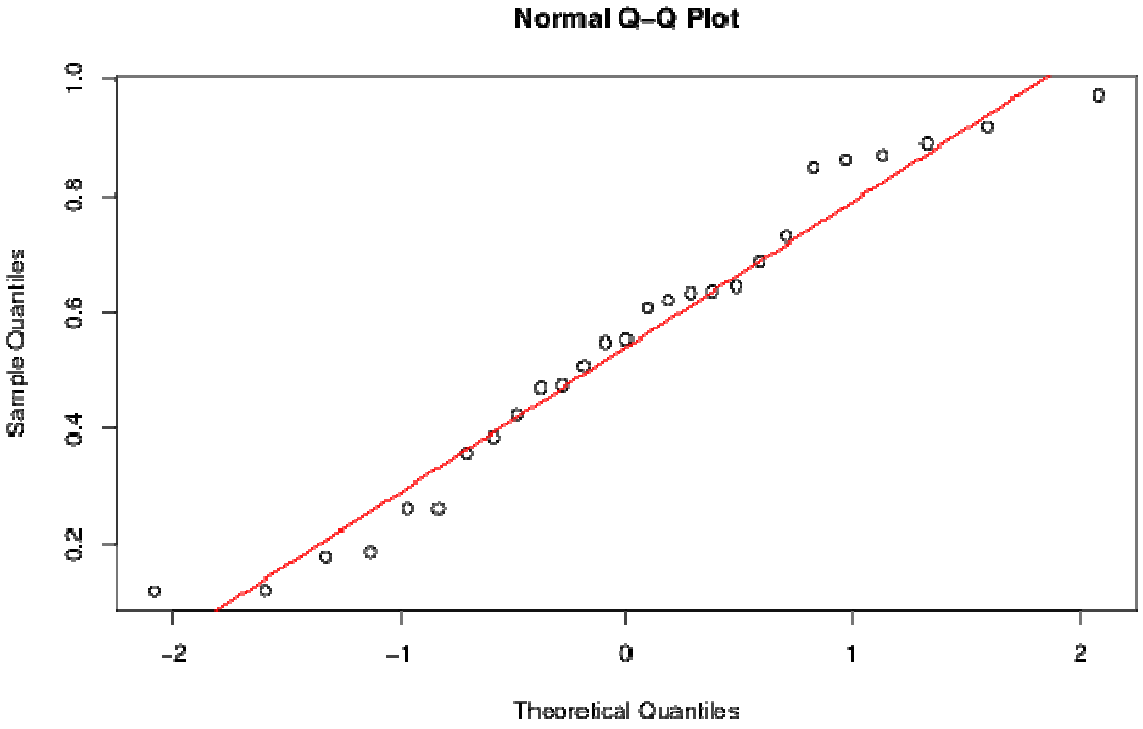


Figure 4. Q-Q Plot of Adjusted Probabilities of Genes for Recurrence Outcome

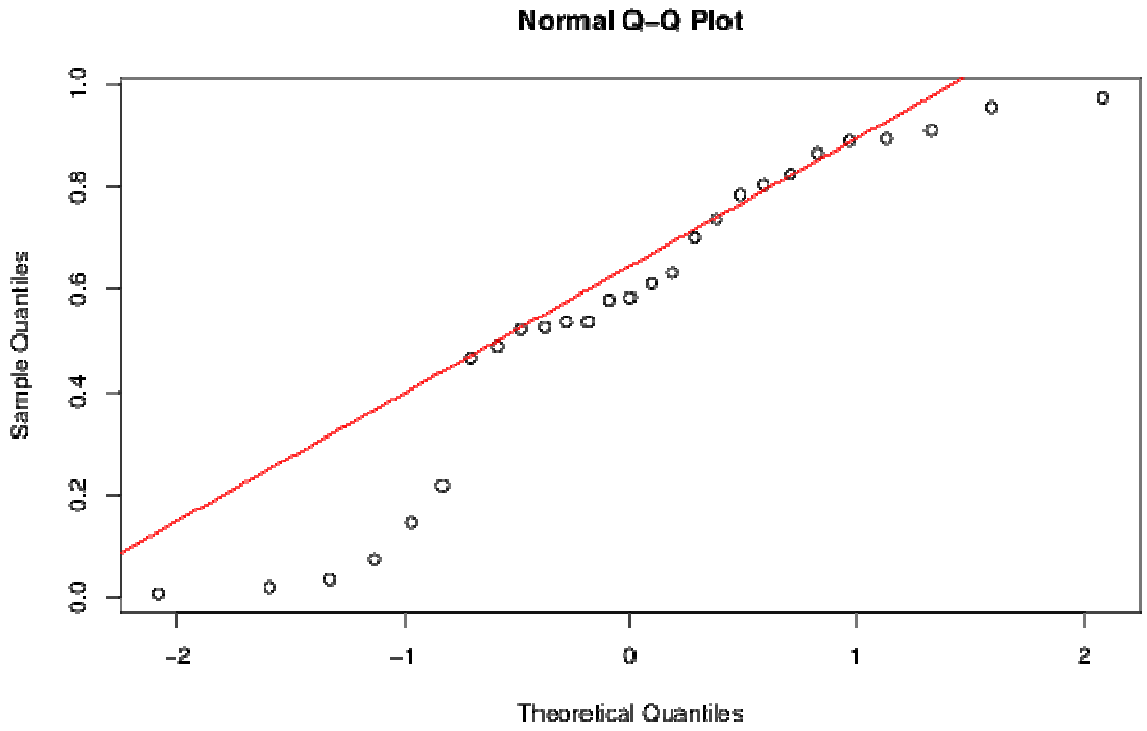


Table 11a. Baseline Demographics, Clinical Characteristics, and RNA Expression Level by Subjects with or without Endpoint of Death among All Melanoma Cases in TCGA dataset (Total N = 194[a])

Baseline Demographics & Clinical Characteristics	Endpoint = Death Event Rate = 85 / 194 (43.8%) Event (N =85)	No Event (N =109)	All Melanoma Cases (N =194)
Gender: n (%)			
- Male	54 (63.5%)	66 (60.5%)	120 (61.9%)
- Female	31 (36.5%)	43 (39.5%)	74 (38.1%)
Age at First Diagnosis (Years):			
Mean ± SD	58.7 ± 15.9	55.1 ± 16.2	56.7 ± 16.2
Stage: n(%)			
- I & I/II	27 (31.8%)	31 (28.4%)	58 (29.9%)
- III & IV	58 (68.2%)	78 (71.6%)	136 (70.1%)
Clark Level: n(%)			
- 1, 2, 3	26 (30.6%)	52 (47.7%)	78 (40.2%)
- 4 & 5	59 (69.4%)	57 (52.3%)	116 (59.8%)
Breslow Index:			
Mean ± SD	4.77 ± 6.89	4.87 ± 8.78	4.82 ± 7.99
RNA Expression Level			
IL-1 RNA Level			
- Continuous (units): Mean ± SD	85.0 ± 178.4	124.3 ± 354.0	107.1 ± 290.5
- Binary: n (%)			
<= 35 units	46 (54.1%)	51 (46.8%)	97 (50.0%)
- > 35 units	39 (45.9%)	58 (53.2%)	97 (50.0%)
ECSIT RNA Level			
- Continuous (units): Mean ± SD	758.8 ± 350.6	724.5 ± 408.6	739.5 ± 383.7
- Binary: n (%)			
<= 670 units	41 (48.2%)	56 (51.4%)	97 (50.0%)
- > 670 units	44 (51.8%)	53 (48.6%)	97 (50.0%)
c-JUN RNA Level			
- Continuous (units): Mean ± SD	2134.5 ± 2180	2001.4 ± 1554	2059.7 ± 1850.4
- Binary: n (%)			
<= 1470 units	44 (51.8%)	54 (49.5%)	98 (50.5%)
- > 1470 units	41 (48.2%)	55 (50.5%)	96 (49.5%)

[a] TCGA dataset was downloaded from the website: https://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp. Among 305 melanoma cases, only 194 subjects with complete data were included.

Table 11b Relationship between RNA Expression Level (as Continuous Variable) and the Melanoma Endpoint of Death from TCGA Dataset (Total N = 194[a]) via Unadjusted and Adjusted Logistic Regression Analysis

Parameter	IL-1 RNA Level		ECSIT RNA Level		c-JUN RNA Level	
	Odds Ratio (95% CI)[b]	p-value	Odds Ratio (95% CI)	p-value	Odds Ratio (95% CI)	p-value
RNA Level (Continuous):						
➢ **Summary Statistics: Mean ±SD**						
- Deaths (N = 85)	85.0 ± 178.4		758.8 ± 350.6		2134.5 ± 2180	
- Survivors (N =109)	124.3 ± 354.0		72453 ±408.6		2001.4 ± 1554	
➢ **Odds ratio for per one unit increase:**						
○ **Unadjusted**	0.999 (0.998, 1.001)	0.3704	1.000 (0.999, 1.001)	0.5364	1.000 (1.000, 1.000)	0.6200
○ **Adjusted**	1.000 (0.998, 1.001)	0.5409	1.000 (0.999, 1.001)	0.6391	1.000 (1.000, 1.000)	0.8167
Covariates:						
- Age at first diagnosis: per one year increase	1.015 (0.996, 1.034)	0.1345	1.015 (0.996, 1.034)	0.1210	1.015 (0.996, 1.034)	0.1262
- Gender: Male vs. Female	1.215 (0.661, 2.232)	0.5306	1.211 (0.650, 2.228)	0.5377	1.200 (0.653, 2.202)	0.5572
- Tumor Stage: III & IV vs. I & I/II	0.546 (0.264, 1.127)	0.1019	0.553 (0.268, 1.142)	0.1092	0.551 (0.267, 1.137)	0.1068
- Clark Level: 4 & 5 vs. 1, 2, & 3	2.513 (1.287, 4.907)	00070*	2.545 (1.304, 4.966)	00062*	2.556 (1.311, 4.984)	00059*
- Breslow Index: Per one unit increase	0.993 (0.956, 1.032)	0.7342	0.991 (0.954, 1.030)	0.6539	0.993 (0.956, 1.032)	0.7328

[a] TCGA dataset was downloaded from the website: https://tcga- data.nci.nih.gov/tcga/tcgaHome2.jsp.
Among 305 melanoma cases, only 194 subjects with complete data were included.

Table 11c. Relationship between RNA Expression Level (as Binary Variable: > vs. <= Median) and the Melanoma Endpoint of Death from TCGA Dataset (N = 194[a]) via Unadjusted and Adjusted Logistic Regression Analysis

Parameter	IL-1 RNA Level: > vs. <= Median: 35 units		ECSIT RNA Level: > vs. <= Median: 670units		c-JUN RNA Level: > vs. <= Median: 1470 units	
	Odds Ratio (95% CI)[b]	p-value	Odds Ratio (95% CI)	p-value	Odds Ratio (95% CI)	p-value
RNA Level (Continuous):						
➢ **Summary Statistics: n / N (%) of > Median**						
- Deaths (N = 85)	39 / 85 (45.9%)		44 / 85 (51.8%)		41 / 85 (48.2%)	
- Survivors (N =109)	58 / 109 (53.2%)		53 / 109 (48.6%)		55 / 109 (50.5%)	
➢ **Odds ratio for per one unit increase:**						
o **Unadjusted**	0.746 (0.422, 1.317)	0.3116	1.134 (0.643, 2.000)	0.6643	0.915 (0.519, 1.614)	0.7587
o **Adjusted**	0.782 (0.428, 1.430)	0.4250	1.083 (0.601, 1.952)	0.6643	0.892 (0.495, 1.607)	0.7038
Covariates:						
- Age at first diagnosis: per one year increase	1.013 (0.994, 1.033)	0.1773	1.015 (0.996, 1.035)	0.1162	1.015 (0.996, 1.035)	0.1150
- Gender: Male vs. Female	1.223 (0.664, 2.252)	0.5178	1.199 (0.654, 2.201)	0.5573	1.196 (0.652, 2.104)	0.5630
- Tumor Stage: III & IV vs. I & I/II	0.544 (0.263, 1.123)	0.0999	0.553 (0.267, 1.144)	0.1104	0.544 (0.264, 1.122)	0.0993
- Clark Level: 4 & 5 vs. 1, 2, & 3	2.581 (1.323, 5.036)	00054*	2.547 (1.305, 4.970)	00061*	2.555 (1.311, 4.981)	00059*
- Breslow Index: Per one unit increase	0.992 (0.955, 1.031)	0.6958	0.993 (0.956, 1.031)	0.7090	0.992 (0.955, 1.031)	0.6907

[a] TCGA dataset was downloaded from the website: https://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp. Among 305 melanoma cases, only 194 subjects with complete data were included.

Table 12a. Baseline Demographics, Clinical Characteristics, and Protein Expression Level by Subjects with or without Endpoint of Death among All Melanoma Cases in TCGA dataset (Total N = 119[a])

	Endpoint = Death Event Rate = 55 / 119 (46.2%)		All Melanoma Cases
Baseline Demographics & Clinical Characteristics	**Event (N =55)**	**No Event (N =64)**	**(N =119)**
Gender: n (%)			
- Male	34 (61.8%)	40 (62.5%)	74 (62.2%)
- Female	21 (38.2%)	24 (37.5%)	45 (37.8%)
Age at First Diagnosis (Years):			
Mean ± SD	60.9 ± 15.1	53.4 ± 15.9	56.9 ± 15.9
Stage: n(%)			
- I & I/II	18 (32.7%)	22 (34.4%)	40 (33.6%)
- III & IV	37 (67.3%)	42 (65.6%)	79 (66.4%)
Clark Level: n(%)			
- 1, 2, 3	17 (30.9%)	29 (45.3%)	46 (38.7%)
- 4 & 5	38 (69.1%)	35 (54.7%)	73 (61.3%)
BreslowIndex:			
Mean ± SD	4.80 ± 7.19	3.70 ± 5.19	4.21 ± 6.20
Protein Expression Level			
c-JUN Protein Level			
- Continuous (units): Mean ± SD	-1.211 ± 0.424	-0.918 ± 0.689	-1.053± 0.598
- Binary: n (%)			
<= -1.12 units	41 (74.5%)	35 (54.7%)	76 (63.9%)
> -1.12 units	14 (25.5%)	29 (45.3%)	43 (36.1%)

[a] TCGA dataset was downloaded from the website: https://tcga-data.nci.nih.gov/tcga/tcgaHome2.jsp. Among 206 cases, only 119 subjects with complete data were included.

Table 12b. Relationship between c-JUN Protein Expression Level (as Continuous Variable) and the Melanoma Endpoint of Death from TCGA Dataset (Total N = 119[a]) via Unadjusted and Adjusted Logistic Regression Analysis

Parameter	c-JUN Protein Expression Level	
	Odds Ratio (95% CI)	**p-value**
c-JUN Protein Level (Continuous):		
➢ **Summary Statistics: Mean ±SD**		
- Deaths (N = 55)	-1.211 ± 0.424	
- Survivors (N =64)	-0.918 ± 0.689	
➢ **Odds ratio for per one unit increase:**		
o **Unadjusted**	0.405 (0.204, 0.804)	0.0098*
o **Adjusted**	0.407 (0.196, 0.844)	0.0156*
Covariates:		
- Age at first diagnosis: per one year increase	1.031(1.005, 1.058)	0.0213*
- Gender: Male vs. Female	0.882 (0.390, 1.994)	0.7631
- Tumor Stage: III & IV vs. I & I/II	0.706 (0.257, 1.937)	0.4992
- Clark Level: 4 & 5 vs. 1, 2, & 3	2.140 (0.848, 5.406)	01074
- Breslow Index: Per one unit increase	1.017 (0.952, 1.086)	0.6262

[a] TCGA dataset was downloaded from the website: https://tcgadata.nci.nih.gov/tcga/tcgaHome2.jsp. Among 206 cases, only 119 subjects with complete data were included.

Table 12c. Relationship between c-JUN Protein Expression Level (as Binary Variable: > vs. <= Median of -1.12 units) and the Melanoma Endpoint of Death from TCGA Dataset (Total N = 119[a]) via Unadjusted and Adjusted Logistic Regression Analysis

Parameter	c-JUN Proein Expression Level	
	Odds Ratio (95% CI)	**p-value**
c-JUN Protein Level (Binary):		
➢ **Summary Statistics: n / N (%) of > Median**		
- Deaths (N = 55)	14 / 55 (25.5%)	
- Survivors (N =64)	29 / 64 (45.3%)	
➢ **Odds ratio for > vs. <= median of -1.12 units:**		
o **Unadjusted**	0.412 (0.189, 0.900)	0.0262*
o **Adjusted**	0.417 (0.182, 0.0.957)	0.0390*
Covariates:		
- Age at first diagnosis: per one year increase	1.031(1.005, 1.058)	0.0211*
- Gender: Male vs. Female	0.823 (0.367, 1.843)	0.6354
- Tumor Stage: III & IV vs. I & I/II	0.631 (0.234, 1.702)	0.3634
- Clark Level: 4 & 5 vs. 1, 2, & 3	2.238 (0.891, 5.622)	0.0864
- Breslow Index: Per one unit increase	1.021 (0.954, 1.093)	0.5444

[a] TCGA dataset was downloaded from the website: https://tcgadata.nci.nih.gov/tcga/tcgaHome2.jsp. Among 206 cases, only 119 subjects with complete data were included.

Chapter 4

Discussion

All the specific SNPs for the upstream and downstream genes in the *IL-1* pathway were specifically selected because they had been selected before in previous studies for showing prior indication of association with human disease, including cancer and its genesis. These studies would include Lee, K.M. et al. (2007), Van Dyke, A.L. et al. (2009), Erdei, E et al. (2010), and Karakus, N. et al. (2011). Regarding the discrepancy in the results for the individual SNP analysis between this study and the French validation, the most likely explanation would be differences in genetic composition between the subjects from the States and those from France, along with certain differences in lifestyle and environmental factors such as exposure to the sun. It is interesting to note that for the composite outcome, r1800587 and rs17561 had hazard ratios with significant p-values and were less than 1, along with the 95% CI. There seems to be an indication that the non-ancestral alleles for these two SNPs might have a protective effect for melanoma outcome and would merit future investigation. Ultimately, more fruitful results were found from the pathway analysis. The results of the individual SNP analysis proved to be intriguing, if inconclusive. One drawback that should be noted is that using the composite outcome of death and/or recurrence would be deemed slightly odd, and that melanoma-specific survival would be more typical.

Regarding the potential protective role *c-JUN* may play in melanoma outcome, Kappelmann et al. (2013) reported that the production of c-JUN protein is regulated at the post-transcription level. There are microRNAs directing the regulation of c-JUN protein expression. This report could explain the lack of significance for *c-JUN* at the RNA level. Kunz (2013) observed that melanoma-associated microRNAs are frequently located in

genomic regions, often showing gains and losses in tumors. However, using logistic regression, c-JUN was found to be significant at the protein level for both unadjusted and adjusted for the key covariates, whether c-JUN was treated as categorical or continuous, with p-values well under 0.05. The same phenomenon might hold true for IL-1 showing significance at the protein level, despite the lack of significance at the RNA level. Due to the lack and limitation of available human data, it will have to wait for a future study.

The pathway analysis showed borderline significance for the *TAK1* gene, but there was no RNA or protein expression data available, but for a future study, it would be useful to consider the reason for these results. Regarding why such results were seen, transforming growth factor-β activated kinase-1 (*TAK1*) is crucial for the cellular response cascade caused by changes in the environment. It is controlled by cytokines including specifically interleukin-1 (*IL-1*) and transforming growth factor-β (*TGF-β*). *TAK1* is also critical for in turn activating key intra-cellular kinases, such p38 MAPK and c-jun N-terminal kinase (*JNK*). Landström (2010) has indicated that *TAK1* is implicated in the activation of tumor suppressor protein, LKB1 kinase. The LKB1 kinase in turn can go on to activate 14 kinases. What is important to note about *TAK1* is that it is acting upstream in all these pathways, further highlighting its key function of *TAK1* in modulating the cellular response to cytokines and stress.

As was mentioned in the introduction, this study did not simply use individual SNP or candidate gene analysis; it implemented a full pathway analysis of all the genes in the *IL-1* pathway, incorporating all available SNPs. The reasons for such are that previous candidate gene studies reported variants in genes related to immunosuppressive mechanisms. The studies focused on some sets of SNPs, but the sample sizes were relatively small, and the

results were at times inconsistent. Analysis of the SNPs of individual genes from this GWAS will thus serve to further elucidate and build upon the results from the previous studies. After the analysis of the individual SNPs, it is intended that a pathway analysis be performed.

Such previous studies can be underpowered in detecting weak associations with susceptibility to disease at the genome-wide significance level. The idea for a pathway analysis is that SNPs in a group of genes with a common biological function might show significant association at the overall pathway level, even if no individual SNP shows association at a proper level of statistical significance. From there, if any genes are found to be significant, statistical analysis of RNA and protein expression levels for those genes will proceed as the final stage of verification, hopefully finding a novel target for therapy.

IL-10, *IL-6*, and *IFN-γ* (Martínez-Escribano, 2002) all have reports involving tumor growth or shrinkage. In this study, these cytokines did not show significant correlation with the melanoma outcomes of death and recurrence (data not shown). The bioinformatical analysis suggests that these cytokines may not be major impact factors for melanoma.

To go further into c-JUN, it combines with c-FOS to form the AP-1 transcription factor. AP-1 is responsible for regulating the expression of genes depending on stimuli, which include cytokines and other things like growth factors and bacterial or viral infections. AP-1 is also responsible for overseeing, among other things, apoptosis and proliferation. *c-JUN* is activated via double phosphorylation of the *JNK* pathway. It is interesting to note that knocking out *c-JUN* causes lethality, but transgenic animal models with a mutated *c-JUN* form that is unable to be phosphorylated are actually viable.

Some other interesting details about *c-JUN* are that it is the transforming gene of avian sarcoma virus 17. *c-JUN* is a gene without introns, located on 1p31-1p32. That

chromosomal region happens to be distinct for its role regarding translocations and deletions influencing diseases. *c-JUN* is positively self-regulated by its own product, which binds to the promoter region to further induce transcription. As for *c-JUN*'s links to other pathways, *c-JUN* activity can be affected by the *ERK* pathway. *ERK* increases transcription of *c-JUN*.

c-JUN is crucial and necessary for the flow of the cell cycle, specifically the G1 phase. Without *c-JUN*, cells show arrest of the G1 phase. Specifically, *c-JUN* is required for cyclin D1 kinase function, which allows the cell cycle to continue flowing.

c-JUN is known for anti-apoptotic activity. Specifically, it can be activated by UV irradiation. *c-JUN* shields cells from apoptosis induced by UV irradiation. It can also work with *NF-κβ* to prevent apoptosis triggered by *TNF-α*.

c-JUN is mainly considered an oncogenic factor in malignant melanoma and many other cancer types (Kappelman et al., 2013). It is surprising to observe that *c-JUN* shows a protective role in melanoma mortality and recurrence. The reason for this discrepancy is not clear. Yamanishi et al. (1991) reported metastatic melanoma cells expressing low *c-JUN* RNA transcript activity. The results of this study correspond with those of Yamanishi et al. (1991). It is possible that the discrepancy could be similar to the observations that *IL-1* shows either pro- or anti-tumor functions depending upon the circumstances. It awaits further clarification.

As to how this discovery might help explain lack of results for drug treatments for melanoma, one example would be *B-RAF* inhibitors such as vemurafenib. *B-RAF* with the V600 mutation activates the *MAPK* pathway, affecting *MEKK1*, which is part of the *IL-1* pathway. However, while *MEKK1* leads down to the activation of *c-JUN*, it is responsible for the other branch of the *IL-1* pathway leading down to the other component of *NF-κβ*. *NF-κβ*

could be connected to other pathways that help it maintain activation despite the disruption of *MEKK1*. That could help explain tumor mechanisms for adapting against the *BRAF* inhibitors. Furthermore, as previously noted, *c-JUN* is positively self-regulating, so once there is already some protein product, *c-JUN* can presumably continue maintaining activation and production even with the upper part of the pathway flowing from *MEKK1* being inhibited.

As far as is known, this study is the first full pathway design correlating *c-JUN* activity with melanoma outcome. The pathway/mechanism for this protective/anti-proliferation effect is unclear. However, using breast cancer as a model, Xu et al. (2013) showed that inhibition of the Tamoxifen-stimulated cells was positively regulated by overexpression of *c-JUN* and vice versa for underexpression of *c-JUN*. The exact mechanism for the anti-proliferation is unknown, though it does occur through the protein kinase C (PKC) pathway. It could be the same for melanoma or perhaps a similar pathway. Based on this bioinformatics analysis, the results suggest that therapeutically targeting *c-JUN* activity may be an effective way to enhance the rate of curing malignant melanoma.

In conclusion, the bioinformatical analyses did not show significant correlation of studied *IL-1*, *Il-6*, *IL-8*, *IFN-γ*, and *TNF-α* SNPs with melanoma mortality, recurrence or composite outcome of both. Full pathway analysis of all SNPs from every gene in the *IL-1* pathway showed that c-JUN protein expression is significantly negatively correlated with mortality and recurrence. The expression and function of *c-JUN* may serve as a therapeutic target for metastatic melanoma.

Bibliography

Alexandrescu DT, Ichim TE, Riordan NH, Marincola FM, Di Nardo A, Kabigting FD, Dasanu CA. Immunotherapy for melanoma: current status and perspectives. J Immunother. 2010 Jul-Aug;33(6):570-90.

Becker, J.K., Goldberg, L.H., Tschen, J.A. Differential diagnosis of malignant melanoma. Am Fam Physician. 1989 May; 39(5): 203-14.

Botella-Estrada, R., Escudero, M., O'Connor, J.E., Nagore, E., Fenollosa, B., Sanmartín, O., et al. Cytokine production by peripheral lymphocytes in melanoma. Eur Cytokine Netw. 2005 Jan-Mar; 16(1): 47-55.

Boyano, M.D., Garcia-Vázquez, M.D., López-Michelena, T., Gardeazabal, J., Bilbao, J., Cañavate, M.L., et al. Soluble interleukin-2 receptor, intercellular adhesion molecule-1 and interleukin-10 serum levels in patients with melanoma. Br J Cancer. 2000 Oct; 83(7): 847-52.

Burns, K., Martinon, F., Esslinger, C., Pahl, H., Schneider, P., Bodmer, J.L., et al. MyD88, an adapter protein involved in interleukin-1 signaling. J Biol Chem. 1998 May 15; 273(20): 12203-9.

Erdei, E., Kang, H., Meisner, A., White, K., Pickett,G., Baca, C., et al. Polymorphisms in cytokine genes and serum cytokine levels among New Mexican women with and without breast cancer. Cytokine. 2010 Jul; 51(1): 18-24.

Estrozi, B., Machado, J., Rodriguez, R., Bacchi, C.E. Clinicopathologic findings and BRAF mutation in cutaneous melanoma in young adults. Diag Mol Pathol 22: 57-64, 2014.

Finger, E.C., Cheng, C.F., Williams, T.R., Rankin, E.B., Bedogni, B., Tachiki, L. et al. CTGF is a therapeutic target for metastatic melanoma. Oncogene. 2013 Feb 25. doi: 10.1038/onc.2013.47.

Garrido F, Algarra I. MHC antigens and tumor escape from immune surveillance. Adv Cancer Res 2001;83: 117–58.

Gray-Schopfer, V., Wellbrock, C., Marais, R. Melanoma biology and new targeted therapy. Nature. 2007 Feb 22; 445(7130): 851-7.

Hersey, P. Zhuang, L., Zhang, X.D. Current strategies in overcoming resistance of cancer cells to apoptosis melanoma as a model. Int Rev Cytol. 2006; 251: 131-58.

Ivanov, V.N., Bhoumik, A., Ronai, Z. Death receptors and melanoma resistance to apoptosis. Oncogene. 2003 May 19;22(20):3152-61.

Kalialis LV, Drzewiecki KT, Klyver H. Spontaneous regression of metastases from melanoma: review of the literature. Melanoma Res. 2009 Oct;19(5):275-82. doi: 10.1097/CMR.0b013e32832eabd5.

Kappelmann, M., Bosserhoff, A., Kuphal, S. AP-1/c-Jun transcription factors: Regulation and function in malignant melanoma. Eur J Cell Biol. 2013 Oct 26. pii: S0171-9335(13)00062-9.

Kappelmann, M., Kuphal, S., Meister, G., Vardimon, L., Bosserhoff, A.K. MicroRNA miR-125b controls melanoma progression by direct regulation of c-Jun protein expression. Oncogene. 2013 Jun 13; 32(24): 2984-91.

Karakus, N., Kara, N., Ulusoy, A.N., Ozaslan, C., Bek, Y. Tumor necrosis factor alpha and beta and interferon gamma gene polymorphisms in Turkish breast cancer patients. DNA Cell Biol. 2011 Jun;30(6):371-7.

Khalili, J.S., Hwu, P., Lizée, G. Forging a link between oncogenic signaling and immunosuppression in melanoma. Oncoimmunology. 2013 Feb 1;2(2):e22745.

Khalili, J.S., Liu, S., Rodríguez-Cruz, T.G., Whittington, M., Wardell, S., Liu, C., et al. Oncogenic BRAF(V600E) promotes stromal cell-mediated immunosuppression via induction of interleukin-1 in melanoma. Clin Cancer Res. 2012 Oct 1;18(19): 5329-40.

Kopp, E., Medzhitov, R., Carothers, J., Xiao, C., Douglas, I., Janeway, C.A., Ghosh, S. ECSIT is an evolutionarily conserved intermediate in the Toll/IL-1 signal transduction pathway. Genes Dev. 1999 Aug 15; 13(16): 2059-71.

Kunz, M. MicroRNAs in melanoma biology. Adv Exp Med Biol. 2013; 774: 103-20.

Landström, M. The TAK1-TRAF6 signalling pathway. Int J Biochem Cell Biol. 2010 May; 42(5): 585-9.

Lee, K.M., Shen, M., Chapman, R.S., Yeager, M., Welch, R., He, X., et al. Polymorphisms in immunoregulatory genes, smoky coal exposure and lung cancer risk in Xuan Wei, China. Carcinogenesis. 2007 Jul; 28(7): 1437-41.

Leong, S.P., Peng, M., Zhou, Y.M., Vaquerano, J.E., Chang, J.W. Cytokine profiles of sentinel lymph nodes draining the primary melanoma. Ann Surg Oncol. 2002 Jan-Feb; 9(1): 82-7.

Martínez-Escribano, J.A., Moya-Quiles, M.R., Muro, M., Montes-Ares, O., Hernández-Caselles, T., Frías, J.F., Alvarez-López, M.R. Interleukin-10, interleukin-6 and interferon-gamma gene polymorphisms in melanoma patients. Melanoma Res. 2002 Oct; 12(5): 465-9.

Mocellin, S., Wang, E., Marincola, F.M. Cytokines and immune response in the tumor microenvironment. J Immunother. 2001 Sep-Oct; 24(5): 392-407.

Nemunaitis, J., Fong, T., Shabe, P., Martineau, D., Ando, D. Comparison of serum interleukin-10 (IL-10) levels between normal volunteers and patients with advanced melanoma. Cancer Invest. 2001;19(3):239-47.

Ocvirk, J. Stabuc, B., Rudolf, Z., Galvani, V., Curin-Serbec, V. Serum values of tumour necrosis factor-alpha and of soluble tumour necrosis factor-R55 in melanoma patients. Melanoma Res. 2000 Jun; 10(3): 253-8.

Pak, B.J., Chu, W., Lu, S.J., Kerbel, R.S., Ben-David, Y. Lineage-specific mechanism of drug and radiation resistance in melanoma mediated by tyrosinase-related protein 2. Cancer Metastasis Rev. 2001;20(1-2):27-32.

Papacleovoulou, G., Critchley, H.O., Hillier, S.G., Mason, J.I.. IL1α and IL4 signalling in human ovarian surface epithelial cells. J Endocrinol. 2011 Dec; 211(3): 273-83.

Porter, G.A., Abdalla, J., Lu, M., Smith, S., Montgomery, D., Grimm, E., et al. Significance of plasma cytokine levels in melanoma patients with histologically negative sentinel lymph nodes. Ann Surg Oncol. 2001 Mar; 8(2): 116-22.

Qin Y, Ekmekcioglu S, Liu P, Duncan LM, Lizée G, Poindexter N, Grimm EA. Constitutive aberrant endogenous interleukin-1 facilitates inflammation and growth in human melanoma. Mol Cancer Res. 2011 Nov;9(11):1537-50.

Segura, M.F., Fontanals-Cirera, B., Gaziel-Sovran, A., Guijarro, M.V., Hanniford, D., Zhang, G., et al. BRD4 sustains melanoma proliferation and represents a new target for epigenetic therapy. Cancer Res. 2013 Oct 15; 73(20): 6264-76.

Singh, R.K., Gutman, M., Reich, R., Bar-Eli, M. Ultraviolet B irradiation promotes tumorigenic and metastatic properties in primary cutaneous melanoma via induction of interleukin 8. Cancer Res. 1995 Aug 15; 55(16): 3669-74.

Van Dyke, A.L., Cote, M.L., Wenzlaff, A.S., Chen, W., Abrams, J., Land, S., et al. Cytokine and cytokine receptor single-nucleotide polymorphisms predict risk for non-small cell lung cancer among women. Cancer Epidemiol Biomarkers Prev. 2009 Jun;18(6):1829-40.

Wu, M.C., Lee, S., Cai, T., Li, Y., Boehnke, M., Lin, X. Rare-variant association testing for sequencing data with the sequence kernel association test. Am J Hum Genet. 2011 Jul 15; 89(1): 82-93.

Xu, Y., Zou, S.T., Zhu, R., Li, W., Gu, C.W., Wei, S.H., et al. Inhibition of proliferation of estrogen receptor‐positive MCF‐7 human breast cancer cells by tamoxifen through c‐Jun transcription factors. Mol Med Rep. 2013 Apr; 7(4): 1283-7.
Yamanishi, D.T., Buckmeier, J.A., Meyskens, F.L., Jr. Expression of c-jun, jun-B, and c-fos proto-oncogenes in human primary melanocytes and metastatic melanomas. J Invest Dermatol. 1991 Aug; 97(2): 349-53.

Zito, C.R., Kluger, H.M. Immunotherapy for metastatic melanoma. J Cell Biochem. 2012 Mar; 113(3): 725-34.

Vita

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1. Wun, I.C. (2014) The Association between Cytokine SNPs And Melanoma Outcome (Ph.D. dissertation; in preparation).

2. Wun, I.C., Amos, C., Frazier, M. (2013) Meta-Analysis of Cholinergic Polymorphisms and Association with Risk for Lung Cancer (manuscript in preparation for submission).

3. Wei C, Han Y, Spitz MR, Wu X, Chancoco H, Akiva P, Rechavi G, Brand H, Wun I, Frazier ML, Amos CI. A case-control study of a sex-specific association between a 15q25 variant and lung cancer risk. Cancer Epidemiol Biomarkers Prev. 20(12):2603-9. doi: 10.1158/1055-9965.EPI-11-0749

4. Ogunjimi, E.O.; Wun, I.C.; Ewens, K.; Wun, W.-S.A.; Huang, J.-C. (2009) Effects of peroxisome proliferator-activated receptor γ activation on human sperm functions. Fertility and Sterility, 92(3), S211.

5. Wun, I.C. (2009) Cholinergic Receptor Candidate Gene Risk Factor for Lung Cancer (master thesis; manuscript submitted)

6. Amos CI, Wei C, Wun I, Dong Q, Spitz MR, Frazier ML. Exploring Rare Variants and Lung Cancer Risk from the Nicotinic Acetylcholine Receptor Region on Chromosome 15q25 in African-Americans, Genetic Epidemiology (33:8), Dec 2009, pp 766-766.

7. Wun, I.C., Dittman, R. (2008). Human Somatic Cell Nuclear Transfer. Chinese J. Physiology, 51(4): 208-213.

8. Wun, I.C., Huang, J.-C. (2008). Enhancement of Sperm Fertilization Capability by Peroxisome Proliferation-Activated Recptor (PPAR) γ Agonist. Submitted to American Society of Reproductive Medicine 64th Annual Meeting in San Francisco, CA.

9. Huang, J.-C., Wun, W.-S.A., Goldsby, J.S., Wun, I.C., Noorhasan, D., Wu, K.K. (2007). Stimulation of embryo hatching and implantation by prostacyclin and peroxisome proliferation-activated receptor delta activation: implication in in vitro fertilization. Human Reproduction, 22, 807 – 814.

10. Huang, J.-C., Wun, W.-S.A., Goldsby, J.S., Wun, I.C., Falconi, S.M., Wu, K.K. (2003). Prostacyclin enhances embryo hatching but not sperm motility. Human Reproduction, 18 (12), 2852 – 2859.

11. J.-C.Huang, W.-S.A.Wun, J.S.Goldsby, I.C.Wun, S.M.Falconi1, and K.K.Wu (2003). Prostacyclin enhances embryo hatching but not sperm motility. Human Reproduction, 18(12), 2582-2589.

12.Huang, J.-C., Wun, W.-S.A., Goldsby, J.S., Wun, I.C., Falconi, S.M., Wu, K.K. Enhanced hatching of mouse embryos by prostacyclin corresponded to the stage-specific expression of prostacyclin receptor by the mouse embryos. Presentation,American Society for Reproductive Medicine, Fifty-Ninth Annual Meeting.

13. Wun, I.C., Herman, K., and Huang, J.-C. Embryonic stem (ES) cell rejuvenate aging mice.(2003). Abstract, Biology of Reproduction 68 (sup.1: 203-204. Presentation in the Program for the Thirty-Sixth Annual Meeting of the Society for the Study of Reproduction, Sabin Cincinnati Convention Center, Cincinnati, Ohio, July 19-22, 2003.