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Prognostic significance of interleukin-6 single nucleotide polymorphism genotypes in neuroblastoma: *rs1800795* (promoter) and *rs8192284* (receptor)

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Abstract

Purpose—Neuroblastoma is a childhood cancer of the sympathetic nervous system and many patients present with high risk disease. Risk stratification, based on pathology and tumor-derived biomarkers, has improved prediction of clinical outcomes, but overall survival rates remain unfavorable and new therapeutic targets are needed. Some studies suggest a link between interleukin-6 and more aggressive behavior in neuroblastoma tumor cells. Therefore, we examined the impact of two IL-6 single nucleotide polymorphisms (SNP) on neuroblastoma disease progression.

Experimental design—DNA samples from 96 high risk neuroblastoma patients were screened for two SNP that are known to regulate the serum levels of IL-6 and the soluble IL-6 receptor (IL-6R), *rs1800795* and *rs8192284* respectively. The genotype for each SNP was determined in a blinded fashion and independent statistical analysis was performed to determine SNP-related event free survival (EFS) and overall survival (OS) rates.

Results—The *rs1800795* IL-6 promoter SNP is an independent prognostic factor for EFS and OS in -high risk neuroblastoma patients. In contrast, the *rs8192284* IL-6 receptor SNP revealed no prognostic value.

Conclusions—The *rs1800795 SNP* (-174 IL-6 (G>C) represents a novel and independent prognostic marker for both EFS and OS in high risk neuroblastoma. Since the *rs1800795 SNP* (-174

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Translational relevance

Recent studies suggest a link between interleukin-6 and more aggressive behavior in neuroblastoma tumor cells. The following study describes the distribution of IL-6 promoter and soluble IL-6 receptor single nucleotide polymorphism (SNP) genotypes in 96 high risk neuroblastoma patients. The study finds that *rs1800795* IL-6 promoter SNP is a novel and independent prognostic factor for outcome in high risk neuroblastoma patients. The clinical relevance of these findings is two-fold. First, the finding that a germ-line SNP in the IL-6 promoter region is associated with neuroblastoma disease progression and survival provides a mechanistic link between host environment and tumor growth potential. Second, since *rs1800795 SNP* (-174 IL-6 (G>C) has been shown to correlate with IL-6 production, this cytokine may represent a target for development of new therapies in neuroblastoma.

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Introduction

Neuroblastoma is the third most common solid tumor in children less than 19 years of age. While neuroblastoma represents less than 10% of all pediatric neoplasms, it is responsible for approximately 15% of all pediatric oncology-related deaths [1]. One of several factors that contribute to poor clinical outcome in neuroblastoma is that over 40% of children with neuroblastoma are categorized as high risk based on the International Neuroblastoma Staging System (INSS) [1]. Despite aggressive intervention, including chemotherapy, surgery, radiation and myeloablative therapy and stem cell rescue, the overall survival rate remains near 30% [1]. Bone is a common site of neuroblastoma metastases, occurring in over half of newly diagnosed patients, and bone metastasis correlates with poor clinical outcomes in neuroblastoma [2].

Metastatic colonization of secondary organs is influenced by interactions between circulating tumor cells and the tissue microenvironments. The bone microenvironment can be a particularly fertile soil for tumor cells due to large repositories of various growth factors. Chemokines generated by these interactions can facilitate not only homing of additional tumor cells but also expanded proliferation and survival of local tumor cell populations. Interleukin 6 (IL-6), a pro-inflammatory cytokine, plays an active role in neoplasia, bone metabolism and iron homeostasis [3, 4]. A role for IL-6 in the disease progression of several neoplasms such as multiple myeloma [5], colon cancer [6], renal cancer [7], Hodgkin's disease [8], non-Hodgkin's lymphoma [9], prostate cancer [10], melanoma [11] and breast cancer [12, 13], has been well documented. Recently, it has been shown that peripheral blood IL-6 levels correlated with disease extent and progression of neuroblastoma [14].

Once in the bone microenvironment, neuroblastoma forms predominantly osteolytic tumors[15]. Recent data suggests that paracrine IL-6 signaling events are involved causing the morbidity associated with neuroblastoma bone metastasis [16]. Bone marrow fibroblasts (i.e. mesenchymal stem cells (MSC)) secrete elevated amounts of IL-6 in the presence of neuroblastoma cells [17, 18], which in turn elevates osteoclast activity and promotes neuroblastoma tumor cell expansion through activation of STAT3 and mitogen-activated protein kinases (MAPK) $p44^{Erk1}$ and $p42^{Erk219}$.

Independent studies from our group found that hormone-responsive breast cancer cells display a similar molecular profile to neuroblastoma cells following paracrine IL-6 stimulation [15, 20], and that breast cancer, like neuroblastoma, has a strong predilection to metastasize to bone [2, 15, 21, 22]. While a link between IL-6 and organ-specific metastasis has yet to be fully elucidated, there exists a direct correlation between the -174 IL-6 (G>C) SNP and clinical outcome in breast cancer. Women with hormone-responsive breast cancer and homozygous "G/G" at the -174 IL-6 (G>C) SNP displayed significantly lower rates of disease free survival (DFS) (P-value < 0.003) and overall survival (OS) (P-value < 0.001) [12].

Given the evidence that elevated levels of circulating IL-6 is a marker of poor prognosis in various cancers, including neuroblastoma, and the role of IL-6 in the neuroblastoma tumor microenvironment; we postulated that a correlation between clinical outcome and IL-6 SNP status may also exist in neuroblastoma patients. Data from this study suggests that increased IL-6 signaling is associated with inferior clinical outcomes in patients with high-risk neuroblastoma patients. This data also suggests that IL-6 may represent a promising extracellular target for new therapies in neuroblastoma.

Materials and Methods

Patient Samples

Ninety-six high risk neuroblastoma DNA specimens collected from blood at the time of diagnosis, with known clinical characteristics and outcome data, were randomly selected from the Neuroblastoma Virtual Tumor Bank. All patients met criteria for COG neuroblastoma high risk group and were enrolled on ANBL00B1. They were subsequently treated on a variety of COG high risk protocol. The patients in this study were enrolled on a trial between June 2, 1994 and January 19, 2007. Inclusion criteria were fulfillment of high risk definition as defined by COG risk grouping at the time of diagnosis, availability of DNA from peripheral blood, and either occurrence of an event, or ≥ 3 years of follow-up time.

Biologic Studies

DNA samples were screened for two SNPs, one of which regulates expression of soluble IL-6 and the other SNP impacts the level of soluble IL-6 receptor (sIL-6R). The SNP in the IL-6 gene promoter -174 IL-6 (G>C) (*rs1800795*) is known to alter expression of IL-6 following inflammatory stimulus [23, 24]. The D358A IL-6 SNP (*rs8192284*) creates an amino acid substitution within the extracellular cleavage domain of the IL-6 receptor (IL-6R) that influences sIL-6R serum levels [24]. We employed restriction fragment length polymorphism (RFLP) mapping to identify each patient as (G/G), (G/C), or (C/C) at the -174 IL-6 (G>C) SNP and (C/C), (A/C), or (A/A) at the D358A IL-6R SNP. The RFLP mapping strategy was previously described by DeMichele, et al. for the -174 IL-6 (G>C) SNP [12] A similar approach was used to assess the SNP genotype for the D358A IL-6R SNP. Both methods were PCR-based and used primers that flank the SNP locus to produce an amplicon of 305 and 524 base pairs, respectively. DNA (0.1 μg) from high risk neuroblastoma patients was used as a template, and PCR amplicons were generated following 35 cycles consisting of: melting 95°C for 2 minutes; annealing 72°C for 1 minute; extension 58°C for 1 minute. The PCR products were gel purified on 2% agarose and digested with the DNA restriction endonuclease *Nla-III* (-174 IL-6 (G>C) SNP) or *Hind-III* (D358A IL-6R SNP). The predicted band sizes for the -174 IL-6 (G>C) SNP genotypes following *Nla-III* digestion were (G/G) = 230bp; (G/C) = 230bp plus 121/109bp; and (C/C) = 121/109bp, and the predicted band sizes following *Hind-III* digestion for the D358A IL-6R SNP genotypes were (A/A) = 413; (A/C) = 413bp plus 231/182bp; (C/C) = 231/182bp (Figure 1). The DNA primer sequences utilized were: "-174 IL-6 (G > C) " *forward*: ATGCCAAGTGCTGAGTCACTA, *reverse*: TCGAGGGCAGAATGAGCCTC, and " D358AIL-6R" *forward*: GGCGCTCAGAAACCCTGAGCT, *reverse*: TGTGTGTGTTGTGGTGTGTGC. Each RFLP analysis included an internal positive control to verify endonuclease activity, which gave rise to products of 75bp for the -174 IL-6 (G>C) SNP and 111bp for the D358A IL-6R SNP (Figure 1). Neuroblastoma cell lines (SK-N-AS, SK-N-DZ, SK-N-FI, SK-N-SH) used in RFLP to compare with study samples (Figure 1) were purchased from American Type Culture Collection (ATCC). These cell lines originated from patients with bone marrow metastases.

Statistical Analysis

A sample size of 70 subjects would be required to detect a 30% difference in three year-event free survival (EFS: 20% to 50%) or overall survival (OS: 30% to 60%) for the genotype G/G compared to G/C or C/C, respectively, in a logrank test with an alpha of 0.05 and 80% power. It is expected that the percentages of each genotype within the general population are as follows: 65% of the -174 IL6 (G>C) genotypes are G/C or C/C and 35% are G/G [21–24]. Therefore, it was estimated that a total of 100 patients would be required to provide about 35 patients with genotype G/G, with the balance of about 65 patients of genotypes G/C or C/C.

Fisher's exact test was used to test for a) associations of the existence of polymorphisms versus each other factor; and, b) differences in the proportion of "G/G" in the -174 IL6 SNP for the general Caucasian population versus the NB cohort. Event-free survival (EFS) time was calculated from the time of enrollment on the front-line or biologic study until the time of the first occurrence of relapse, progressive disease, secondary malignancy, or death, or until the time of last contact if no event occurred. Overall survival (OS) time was calculated from the time of enrollment on study until the time of the death, or until last contact with patient. The median follow-up time for patients alive without an event was 4.9 years (range: 3.0 to 11.4 years). The methods of Kaplan-Meier were used to generate survival curves, and curves were compared using a logrank test. EFS and OS are presented as the estimate +/− the standard error, with standard errors calculated per the methods of Peto[25]. The Cox proportional hazards regression model was used to test for the independent predictive ability of the existence of polymorphisms after adjustment for other significant factors. P-values <0.05 were considered statistically significant.

A secondary analysis was performed comparing G/G vs (G/C or C/C) of the promoter -174 IL-6 (G>C) SNP, where (G/C or C/C – "any C") was hypothesized to have better outcome than G/G. The receptor D358A IL-6R SNP was grouped as C/C or A/C ("any C"), which correlates with lower sIL-6R serum levels[26] (hypothesized "better" outcome), versus A/A (hypothesized "worse" outcome). The "better" outcome patients based on the -174 IL-6 (G>C) SNP were combined with the "better" outcome patients based on the D358A IL-6R SNP, and the EFS and OS were compared to the "worse" outcome patients from each SNP. Cross tabulation of the -174 IL-6 (G>C) SNP versus the D358A IL-6R SNP was performed.

Results

IL-6 SNP genotypes in neuroblastoma patients

The characteristics of high risk neuroblastoma patient sample set compared to known risk factors for high-risk disease are shown on Table 1. To determine the genotype of the two SNP known to have the greatest impact on serum levels of IL-6 (-174 IL-6 (G>C); *rs1800795*) and the soluble IL-6 receptor (D358A IL-6R; *rs8192284*), RFLP analysis was performed on 4 neuroblastoma tumor cell lines and 96 DNA samples from high risk neuroblastoma patients (Figure 1). Each RFLP analysis included an internal positive control to verify endonuclease activity, which gave rise to products of 75bp for the -174 IL-6 (G>C) SNP and 111bp for the D358A IL-6R SNP (Figure 1). While the allele frequencies of the D358A sIL-6R SNP were similar to those reported previously [27], the frequency of the -174 IL-6 (G>C) SNP was skewed by 54% toward the G/G genotype relative to published population genotypes [28–30] (Table 2). The proportion of patients in this high risk neuroblastoma cohort with the G/G genotype (57.3%) was statistically higher (P-value = 0.0003) than that of the general Caucasian population (37.1%, i.e., 299 of 807 [28–30]). Caucasians are highly polymorphic at the -174 IL-6 (G>C) SNP while African-Americans and Asians are predominantly G/G at this locus [28–30]. Therefore, we examined whether the observed shift toward G/G at the -174 IL-6 (G>C) SNP in high risk neuroblastoma patients was due to the racial makeup of our random sample. Of the 96 samples that SNP data was obtained, 62.5%, 19.8%, 5.2%, and 12.5% were classified as White/Caucasian, Black/African-American, Asian/South-East Islander, and unknown/other, respectively. A similar skewing toward the G/G genotype at the -174 IL-6 (G>C) SNP was observed for the Caucasian subset (G/G = 53.3%, G/C = 38.3%, C/C = 8.3%), suggesting that differences in racial polymorphic frequencies did not influence the observed shift from previous population studies.

Impact of IL-6 SNP biomarkers on EFS and OS in neuroblastoma

To determine if the -174 IL-6 (G>C) SNP and the D358A IL-6R SNP were predictive of neuroblastoma disease progression and mortality, we examined 3-year EFS and OS rates within the 96 genotyped DNA patient samples. Pairwise statistical analysis was completed for each of the three genotypes within both IL-6-related SNP, and further secondary comparisons were performed across each of the two SNP genotypes. Only the -174 IL-6 (G>C) SNP demonstrated statistically significant differences (P-value < 0.05) in predicting EFS and OS rates (Figure 2A and 2B). Patients carrying one or more C alleles had a higher OS than those who are homozygous for the G allele (3-year OS: 56% ± 8% versus 45% ± 7%; p=0.0413). Secondary analysis of EFS and OS curves of "better" versus "worse" outcome between the -174 IL6 (G>C) and D358A IL-6R SNP revealed no statistically significant differences (data not shown), which was not surprising since the D358A IL-6R polymorphisms were themselves not statistically significantly different (Figure 2C and 2D). Next, we determined whether the -174 IL-6 (G>C) SNP represented a novel, independent genetic biomarker for neuroblastoma. Current risk stratification for neuroblastoma includes age, INSS stage, *MYCN* status, INPC Histology, and DNA ploidy [1]. In addition, 11q LOH, and 1p deletion are established adverse cytogenetic markers in neuroblastoma [1, 31]. No statistically significant associations were found between the -174 IL-6 (G>C) SNP and any of these neuroblastoma risk stratification factors (Table 3). Interestingly, neuroblastoma patients with at least one C allele (i.e., any C) at the -174 IL-6 (G>C) SNP exhibited significantly better EFS and OS rates (Figure 2A and 2B). The -174 IL-6 (G>C) SNP remained independently prognostic after adjustment for *MYCN* amplification (Model B) or after adjustment for diploidy (Model C) (Table 3).

Discussion

We have demonstrated that the -174 IL-6 (G>C) SNP is an independent prognostic marker for clinical outcome in high risk neuroblastoma patients (Table 3 and Figure 2A and 2B). In contrast to the -174 IL-6 (G>C) SNP located in the IL-6 promoter, the D358A IL-6R receptor SNP failed to demonstrate any association between high risk neuroblastoma and disease progression or survival (Figure 2C and 2D).

In the Caucasian population, the published distribution of G/G, G/C and C/C polymorphisms at the -174 IL-6 (G>C) SNP promoter region is 37.1%, 45.6% and 17.3%, respectively [26–29]. Individuals who harbor the homozygous G/G polymorphism produce IL-6 concentrations of [5.35 ± 3.01 pg/L] compared with those carrying G/C [3.96 ± 2.71 pg/ml] or C/C [3.52 ± 2.4 pg/ml] (G/G versus C/C, P-value < 0.05) [29] and other studies support a similar association between the G allele and elevated serum IL-6 levels in healthy volunteers [23, 24]. Data from several studies have demonstrated that the -174 IL-6 (G>C) SNP results in a functional alteration which affects gene transcription and subsequent serum levels of IL-6 cytokine [23, 24, 26–28, 32–35]. These data support the use of the -174 IL-6 (G>C) SNP genotype as a surrogate marker of cumulative IL-6 exposure. In our population of high risk neuroblastoma patients, 57.3% were carriers of the G/G polymorphism (Table 1), and the IL-6 promoter polymorphism identified two subsets of high risk neuroblastoma patients (Figure 2). In the first subset, individuals carrying one or more C alleles had a 3-year overall survival of 56% ± 8%, while those who are homozygous for the G allele had a lower overall survival, 45% ± 7% at 3-years (Figure 2B). As shown recently by Egler et al [14], patients with elevated IL-6 have significantly decreased EFS. The consequent elevated production of IL-6 in individuals with G/G SNP in the IL6 promoter region may be a plausible explanation for these data, suggesting that IL-6 may be a factor involved in neuroblastoma disease progression. Although elevated IL-6 level may be the consequence of advanced disease, our data suggest that the genetic makeup of the individual could also play a role in neuroblastoma disease progression. The correlation between IL-6 level, advanced disease and poor outcome may be part of a neuroblastoma-stimulated

proliferation loop, similar to the one seen in multiple myeloma. Interestingly, stable physiological differences in the soluble IL-6 receptor due to the D358A sIL-6R polymorphism (e.g., A/A = 23.8 ng/ml, A/C = 29.7 ng/ml, C/C = 39.7 ng/[ml][26]), did not impact clinical outcomes within the same patient population suggesting that elevated sIL6-R levels are not compensatory during limited bioavailability of IL-6 (Figure 2).

Neuroblastoma is a biologically heterogeneous tumor, with a spectrum of clinical presentations and responsiveness to therapy. Despite the ability to identify high-risk disease by numerous adverse prognostic biomarkers for neuroblastoma, the overall survival of high risk patients remains poor [1]. The identification of yet another biomarker of inferior outcome in high-risk neuroblastoma in itself adds little. However, there is growing evidence that suggests that IL-6 may plays a role in neuroblastoma growth and dissemination. Retinoic acid has been shown to down modulate the expression of IL-6 receptor α chain [36, 37], inhibit secretion of IL-6 by stromal cells [37], and disrupt the IL-6 autocrine signaling pathway [38, 39]. In CCG3891, it was demonstrated that the addition of cis-retinoic acid during the post-transplant consolidation phase of therapy was beneficial with improved EFS in high risk neuroblastoma patients [40]. It is felt the primary benefit of cis-retinoic acid is inducing terminal differentiation in neuroblastoma cells; however, the role of cis-retinoic acid may also function by down regulating IL-6 signaling events, a hypothesis worthy of further investigation. Additionally, IL-6 can now be targeted therapeutically anti-IL-6 monoclonal antibodies which are commercially available (e.g., CNTO 328, Tocilizumab®).

Additional retrospective and prospective studies are underway to expand on our current data and to elucidate the functional consequences of the -174 IL-6 (G>C) promoter polymorphism within the entire neuroblastoma patient population. Data from this study adds to the growing literature of the association between the -174 IL-6 (G>C) SNP and clinical outcomes of various cancers. [11, 12, 20, 21]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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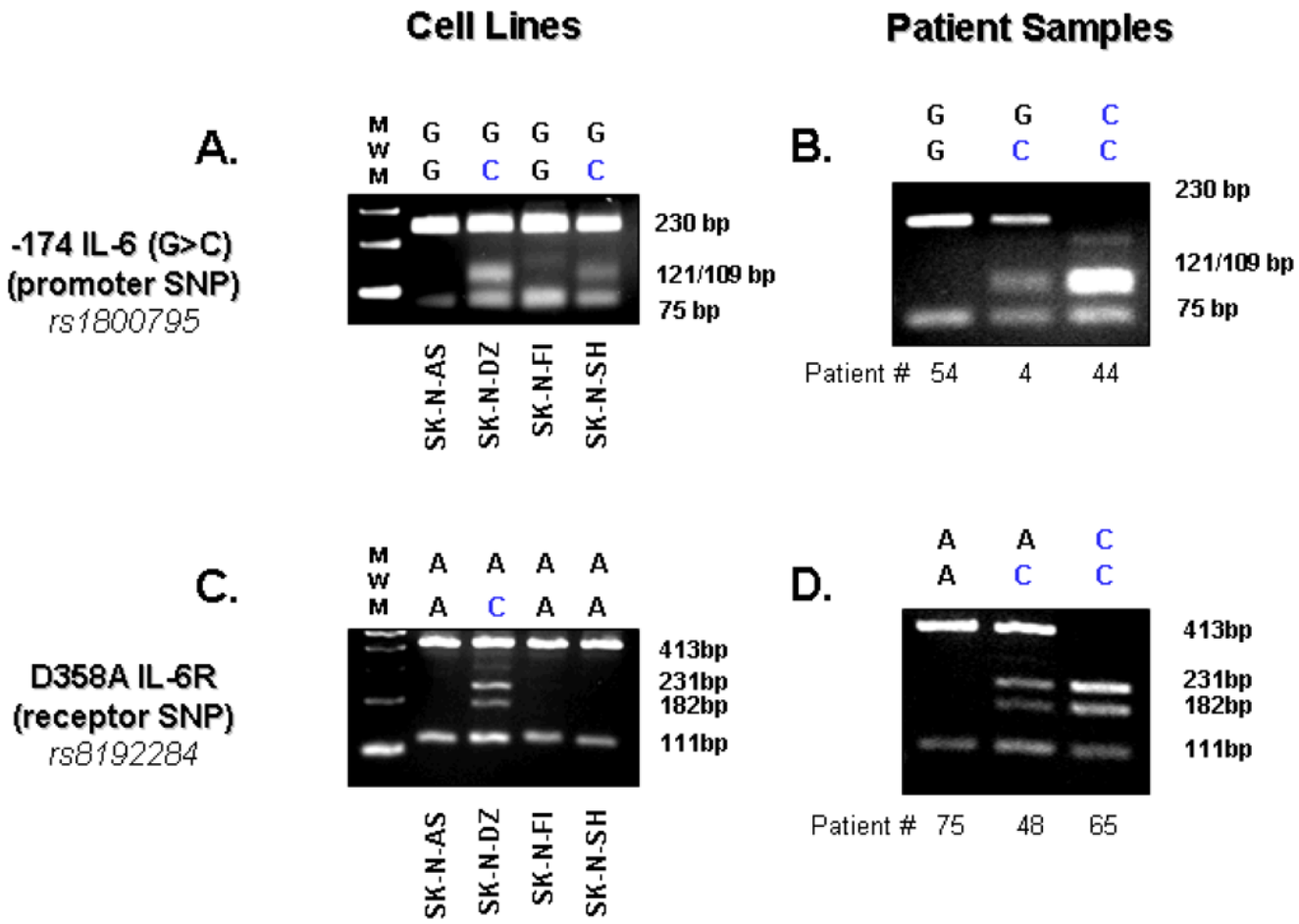

Figure 1.

Restriction Fragment Length Polymorphism (RFLP) analysis of human neuroblastoma (NBL) cell lines and patient samples. RFLP analysis was used to identify the genotypes of the *rs1800795* SNP (-174 IL-6 (G>C)) in (A) human NBL cell lines and (B) high risk NBL patient DNA samples, and in a similar fashion, the genotypes of the *rs8192284* SNP (D358A IL-6R) in (C) human NBL cell lines and (D) high risk NBL patient DNA samples were also characterized. See Table 2 for full genotype analysis.

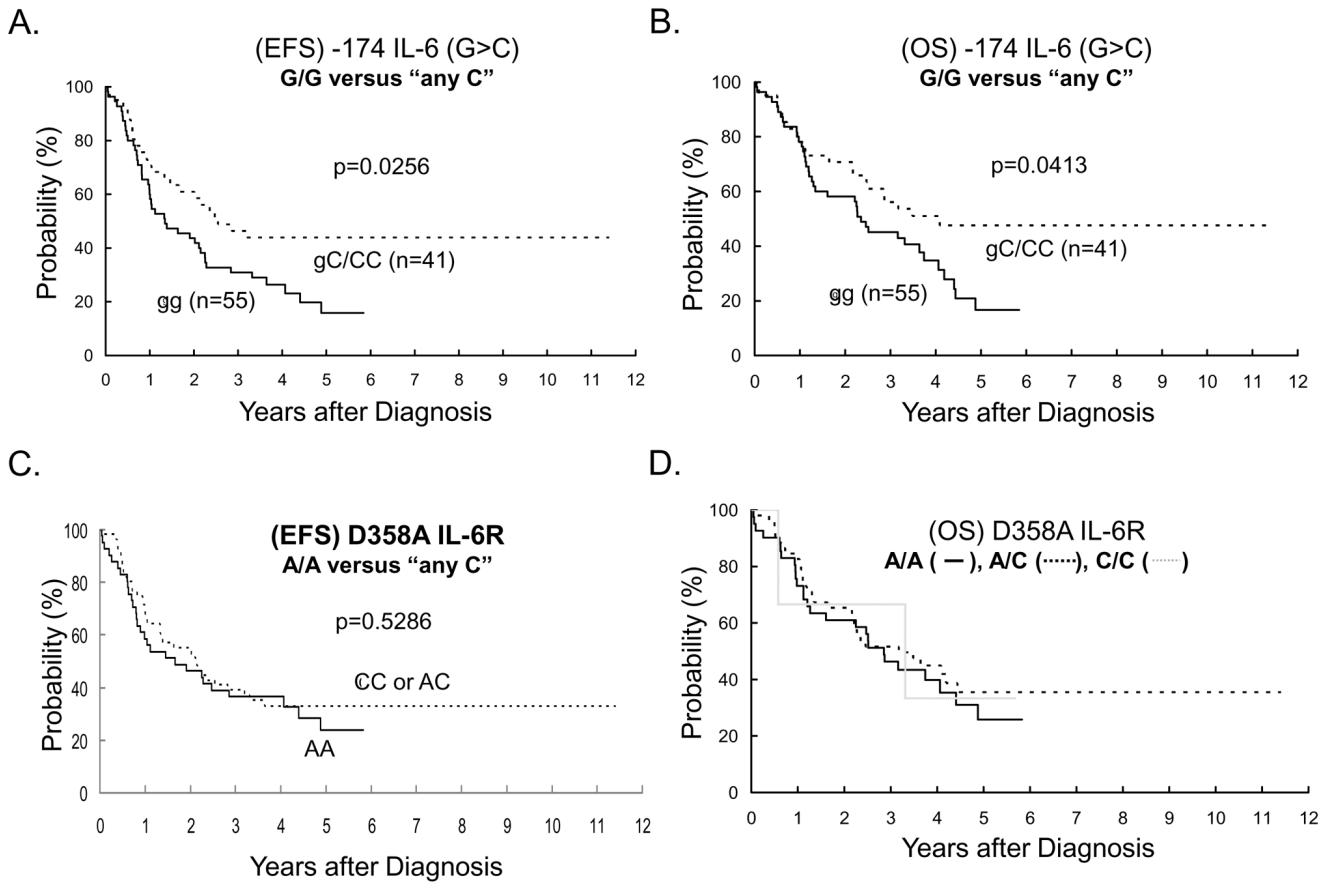

Figure 2.

Kaplan-Meier curves for event-free (EFS) and overall survival (OS) rates. EFS and OS rates were compared between G/G and "any C" (G/C or C/C) for the IL-6 promoter (*rs1800795*) SNP and between A/A and "any C" (A/C or C/C) for the IL-6 receptor (*rs8192284*) SNP.

Table 1

Characteristics of high risk neuroblastoma patient sample set (n = 96) compared to known risk factors.

Factor	N (%)	-174 IL-6 (G>C)			
		(G/G)	(G/C)	(C/C)	Unknown
Age					
< 365 days	12 (12%)	7	4	1	0
365–547 days	7 (7%)	5	1	0	1
≥ 548 days	79 (81%)	43	28	7	1
Sex					
Male	58 (59%)	35	18	4	1
Female	40 (41%)	20	15	4	1
INSS Stage *(*all patient samples were COG High Risk)*					
Stage 1	0 (0%)	0	0	0	0
Stage 2A/2B	1 (1%)	1	0	0	0
Stage 3	17 (17%)	8	7	2	0
Stage 4	79 (81%)	46	25	6	2
Stage 4s	1 (1%)	0	1	0	0
MYCN amplification					
Amplified	40 (43%)	25	13	2	0
Not amplified	53 (57%)	26	19	6	2
Unknown	5	4	1	0	0
Histology (Shimada)					
Favorable	6 (8%)	3	1	1	1
Unfavorable	74 (92%)	40	27	6	1
Unknown	18	12	5	1	0
11q LOH					
Present	26 (43%)	15	8	3	0
Absent	34 (57%)	16	13	3	2
Unknown	38	24	12	2	0
1p deletion					
Present	27 (44%)	14	10	3	0
Absent	34 (56%)	19	10	4	1

Clin Cancer Res. Author manuscript; available in PMC 2010 August 15.

Factor	N (%)	-174 IL-6 (G>C)			
		(G/G)	(G/C)	(C/C)	Unknown
Unknown	37	22	13	1	1

Table 2

Allele frequencies for *rs1800795* (IL-6 promoter) and *rs8192284* (IL-6 receptor) SNP in high risk neuroblastoma (NBL) patients. Allele frequencies of two SNP known to impact IL-6 signaling events were compared between high risk NBL patients and those published in other population-based studies. While similar frequencies were noted between the IL-6 receptor SNP (*rs8192284*), a statistically significant shift toward G/G was observed the promoter SNP (*rs1800795*) in high risk NBL patients compared to the general population (P-value = 0.0003; *see text for more details*).

	Allele Frequencies	
Genotype	**High Risk Neuroblastoma Samples (%)**	**Population (%) (ref: *28–30; †27)**
-174 IL-6 (G>C) (*rs1800795*)		
G/G	55 (57.3)	*37.1
G/C	33 (34.4)	*45.2
C/C	8 (8.3)	*17.7
D358A IL6R (*rs8192284*)		
A/A	41 (42.7)	†34.5
A/C	52 (54.2)	†49.7
C/C	3 (3.1)	†15.8

Table 3

Test for independent statistical significance of -174 IL6 SNP after adjustment for NB risk factors

Factor	N (%)	-174 IL-6 (G>C)			
		(G/G)	(G/C)	(C/C)	Unknown
Age					
< 365 days	**12 (12%)**	7	4	1	0
365–547 days	**7 (7%)**	5	1	0	1
≥ 548 days	**79 (81%)**	43	28	7	1
Sex					
Male	**58 (59%)**	35	18	4	1
Female	**40 (41%)**	20	15	4	1
INSS Stage *(*all patient samples were COG High Risk)*					
Stage 1	**0 (0%)**	0	0	0	0
Stage 2A/2B	**1 (1%)**	1	0	0	0
Stage 3	**17 (17%)**	8	7	2	0
Stage 4	**79 (81%)**	46	25	6	2
Stage 4s	**1 (1%)**	0	1	0	0
***MYCN* amplification**					
Amplified	**40 (43%)**	25	13	2	0
Not amplified	**53 (57%)**	26	19	6	2
Unknown	**5**	4	1	0	0
Histology (Shimada)					
Favorable	**6 (8%)**	3	1	1	1
Unfavorable	**74 (92%)**	40	27	6	1
Unknown	**18**	12	5	1	0
11q LOH					
Present	**26 (43%)**	15	8	3	0
Absent	**34 (57%)**	16	13	3	2
Unknown	**38**	24	12	2	0
1p deletion					
Present	**27 (44%)**	14	10	3	0
Absent	**34 (56%)**	19	10	4	1

Factor	N (%)	-174 IL-6 (G>C)			
		(G/G)	(G/C)	(C/C)	Unknown
Unknown	37	22	13	1	1