Global Assessment of Genetic Variation Influencing Response to Retinoid Chemoprevention in Head and Neck Cancer Patients

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Abstract
Head and neck squamous cell carcinoma (HNSCC) patients are at an increased risk of developing a second primary tumor (SPT) or recurrence following curative treatment. 13-cis-retinoic acid (13-cRA) has been tested in chemoprevention clinical trials, but the results have been inconclusive. We genotyped 9,465 single nucleotide polymorphisms (SNP) in 450 patients from the Retinoid Head and Neck Second Primary Trial. SNPs were analyzed for associations with SPT/recurrence in patients receiving placebo to identify prognosis markers and further analyzed for effects of 13-cRA in patients with these prognostic loci. Thirteen loci identified a majority subgroup of patients at a high risk of SPT/recurrence and in whom 13-cRA was protective. Patients carrying the common genotype of rs3118570 in the retinoid X receptor (RXRA) were at a 3.33-fold increased risk (95% CI, 1.67–6.67) and represented more than 70% of the study population. This locus also identified individuals who received benefit from chemoprevention with a 38% reduced risk (95% CI, 0.43–0.90). Analyses of cumulative effect and potential gene–gene interactions also implicated CDC25C:rs6596428 and JAK2:rs1887427 as 2 other genetic loci with major roles in prognosis and 13-cRA response. Patients with all 3 common genotypes had a 76% reduction in SPT/recurrence (95% CI, 0.093–0.64) following 13-cRA chemoprevention. Carriers of these common genotypes constituted a substantial percentage of the study population, indicating that a pharmacogenetic approach could help select patients for 13-cRA chemoprevention. The lack of any alternatives for reducing risk in these patients highlights the need for future clinical trials to prospectively validate our findings. Cancer Prev Res; 4(2); 185–93. ©2011 AACR.

Introduction
More than 900,000 individuals are diagnosed with head and neck squamous cell carcinoma (HNSCC) each year worldwide, and almost half of them eventually succumb to this disease (1–3). Early-stage HNSCC patients are often treated with surgery and/or radiation therapy in attempt to cure their disease (4); however, second primary tumors (SPT; 15%–25% within 5 years) and/or local-regional recurrence (10% within 5 years) in these curatively treated patients develop frequently and pose a major threat to long-term survival (5, 6). Therefore, the identification and development of novel biomarkers that can predict SPT/recurrence in these patients would allow for intensive surveillance or targeted chemoprevention in those high-risk patients while reducing the burden of a dismal prognosis.

Retinoids, vitamin A and, natural and synthetic derivatives of vitamin A, are the best studied of many agents that have been evaluated for preventing SPT/recurrence of HNSCC (7, 8). It is thought that the chemopreventive functions of retinoids are mediated by binding to and the subsequent activation of specific retinoic acid receptors (RAR) and retinoid X receptors (RXR), each of which exists in several subtypes designated α, β, or γ (9, 10). After their synthesis in the cytoplasm, the RARs and RXRs are translocated into the cell nucleus, where they form various homodimers and heterodimers, which bind to consensus DNA sequences in regulatory regions of target genes and recruit coactivators to regulate the transcription of a wide array of genes that play important roles in regulation of cell growth, differentiation, survival, and transformation (10). Retinoid receptors can also suppress gene expression by antagonizing other nuclear factors. For instance, retinoid treatment has been shown to...
inhibit the activator protein-1-mediated aberrant squamous cell differentiation and sensitize squamous carcinoma cells to chemotherapeutic agents (11, 12). In addition, retinoids induce growth arrest and cellular differentiation associated with retinoblastoma hypophosphorylation through mitogen-activated protein kinases signaling (10, 13). Retinoids have also been reported to induce G1 cell cycle arrest through the activation of proteasome-dependent proteolysis of key proteins such as cyclin D1 (CCND1) and the G2 cyclins (7, 14–16).

One of the best-studied retinoids in cancer chemoprevention is 13-cis-retinoic acid (13-cRA, or isotretinoin), a naturally occurring retinoid which does not bind nor activates nuclear retinoid receptors unlike all-trans-retinoic acid (ATRA) or 9-cis-retinoic acid (9-cRA) but has a more favorable pharmacodynamic properties (17). It has been proposed that the potent antitumor action of 13-cRA is at least partially through conversion to its more transcriptionally active isomers such as ATRA and 9-cRA (17–19). In addition, 13-cRA may also regulate cell growth through binding to nonretinoid membrane receptors such as the mannose-6-phosphate/insulin–like growth factor II receptor (17, 20). Hong and colleagues reported that high-dose 13-cRA given for 1 year significantly reduced the incidence of SPT in curatively treated stages I to IV HNSCC patients and that this effect may last for several years (21, 22). However, in a subsequent large-scale phase III clinical trial of low-dose 13-cRA involving 1,190 randomized, eligible stage-I and stage-II HNSCC patients, no difference in the SPT and/or recurrence rate was observed between the 13-cRA and placebo arms (23). These conflicting results led us to question whether or not a patient’s genetic background may influence the risk of developing SPT/recurrence, and whether these markers could be used to predict which patients are most likely to benefit from 13-cRA.

In this study, we assessed genetic variation via genotyping nearly 10,000 single nucleotide polymorphisms (SNP) from cellular pathways related to cancer in 450 patients recruited to a large-scale, randomized phase-III prevention trial of low-dose 13-cRA (30 mg/d) or placebo in early-stage head and neck cancer patients. First, we assessed the SNPs as prognostic markers, or for associations with the risk of SPT/recurrence in patients who received placebo. Second, we assessed the SNPs as predictive markers, or for associations with the development of SPT/recurrence in patients who received 13-cRA compared to patients with the same genetic profile who received placebo.

Methods

Study population and epidemiologic data

HNSCC patients for this study were a cohort from a large-scale, phase-III prevention trial in 1,190 randomized, eligible patients (1991–1999). The primary end point of this trial was to assess the chemoprevention effect of a daily low dose (30 mg/d for 3 years) of 13-cRA on SPT/recurrence of early-stage HNSCC patients who had been successfully treated with surgery and/or radiation. This study design has been described previously in detail (5, 23). Briefly, HNSCC patients were recruited from The University of Texas MD Anderson Cancer Center, the Radiation Therapy Oncology Group, the Clinical Community Oncology Group, and the Southwest Oncology Group. Histopathologically confirmed early-stage (stages I and II) HNSCC patients, who had remained cancer-free for at least 16 weeks after the end of treatment at the time of recruitment, were randomized to receive either 13-cRA or placebo for a total of 3 years. Information on SPT/recurrence was collected at 3, 6, 9, 12, 16, 20, 24, 28, 32, and 36 months postrandomization with additional evaluations at 6 month intervals for 4 years following intervention. SPT was defined as one of the following: (i) diagnosis of a new cancer with a different histologic type, (ii) cancer of identical histologic type appearing more than 3 years following treatment of the primary tumor, or (iii) discovery of a cancer separated from the primary tumor site by more than 2 cm of clinically normal epithelium. Recurrence was defined as any tumor of similar histology occurring within 2 cm or 3 years of the primary tumor. Written informed consent was obtained from all participants and this study was approved by the University of Texas MD Anderson Cancer Center’s Institutional Review Board.

Genotyping

Genomic DNA was extracted from peripheral blood lymphocytes for each study participant and stored at −80°C until use. We genotyped SNPs in patient DNA samples using Illumina's iSelect genotyping platform following the Infinium II protocol with genotypes called using the BeadStudio software. A combination of haplotype tagging and candidate functional SNPs were selected from genes in 12-cellular pathways as described in Wu and colleagues (24). All genotyping was done blinded to patient outcome status and intervention arm. We applied quality-control procedures in identifying the number of SNPs and patients that could be analyzed.

Statistical analysis

Statistical analyses were done using STATA software (version 10; STATA Corporation). For comparison of the 13-cRA and placebo treatment groups, we used χ² analysis to evaluate the differences in categorical variables and the Student’s t test to assess continuous variables. A former smoker was a person who had quit smoking at least 1 year prior to diagnosis, whereas a current smoker was someone who was currently smoking or who had stopped within the 1 year prior to being diagnosed. The risk of SPT/recurrence was estimated as HR and 95%CI using the multivariable Cox proportional hazard regression model adjusted for age, gender, ethnicity, smoking status, tumor site, and tumor stage. Bootstrap resampling was done 10,000 times to internally validate the results from our analyses. Survival tree analysis using the Stree program (ref. 25; http://c2s2.yale.edu/software/stree/) was used to identify potential higher-order gene–gene interactions among the candidate prognostic SNPs. Terminal nodes were
grouped on the basis of quartiles for percentage of events in that subgroup. Kaplan–Meier curves and corresponding log-rank tests were used to compare event-free (SPT/recurrence) durations between different groups. All P values were 2-sided and P < 0.05 was considered the threshold for statistical significance.

Results

Patient characteristics

Samples of 450 patients were available for SNP genotyping. Other than a higher percentage of Caucasians in the 450 patients versus the remaining 740 patients of the parent trial (95% versus 89%; P = 0.001), there were no significant differences between these 2 groups in patient characteristics such as age, gender, smoking, alcohol, tumor site, stage, radiotherapy, surgery, or 13-cRA treatment. Of the 450 patients, 10 were excluded for quality-control issues, leaving 440 patients in our present study. Approximately half (225, or 51.1%) of the 440 patients received low-dose 13-cRA. Randomization of patients into treatment arms was successful with no significant differences between these 2 groups in patient characteristics (t-test). Other than a higher percentage of Caucasians in the placebo arm at a high risk of developing SPT/recurrence, compared with patients who had variant containing genotypes (Table 2).

Identification of patients in the placebo arm at a high risk of developing SPT/recurrence

We genotyped a total of 9,456 SNPs in the full group of 440 patients and analyzed the effect of genetic variation on SPT/recurrence risk in those randomized to the placebo-only arm to identify potential prognostic markers. A total of 159 SNPs were significantly associated (P < 0.01) with altered risk with the common (wild-type) genotype resulting in an increase in risk for 45 of these loci (Supplementary Table S1). We focused on these common risk genotypes because they identified the majority of the population who would be potential candidates for 13-cRA chemoprevention.

The strongest association with increased SPT/recurrence risk was for patients with the common genotype for the RXRA SNP rs3118570, which represented 71.2% (n = 153) of the study population in the placebo group. These individuals were at a 3.33-fold increased risk of SPT/recurrence (95% CI, 1.67–6.67), and had significant reduction in event-free survival by more than 34 months (P = 0.0002) compared with those with at least one variant rs3118570 allele (Fig. 1A). Other highly significant associations (P < 0.005) were observed for common genotypes in Janus kinase 2 (JAK2), matrix metallopeptidase 3 (MMP3), RAD54-like (RAD54L), and cell division cycle 25 homolog C (CDC25C). Patients with these common genotypes were 1.85 to 3.57 times more likely to develop SPT/recurrence, compared with patients who had variant containing genotypes (Table 2).

Interestingly, in a converse analysis, the top 27 SNPs significantly associated (P < 0.001) with SPT/recurrence in the 13-cRA arm showed no effect on risk for patients receiving placebo (Supplementary Table S2). Even though

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### Table 1. Study population characteristics

<table>
<thead>
<tr>
<th>Variables</th>
<th>Placebo, n (%)</th>
<th>13-cRA, n (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total patients</td>
<td>215 (48.9)</td>
<td>225 (51.1)</td>
<td>0.60</td>
</tr>
<tr>
<td>Age, mean (SD)</td>
<td>60.89 (10.57)</td>
<td>61.40 (9.95)</td>
<td>0.60</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>164 (76.28)</td>
<td>186 (82.67)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>51 (23.72)</td>
<td>39 (17.33)</td>
<td>0.10</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>206 (95.81)</td>
<td>217 (96.44)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>9 (4.19)</td>
<td>8 (3.56)</td>
<td>0.73</td>
</tr>
<tr>
<td>Smoking Status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>34 (15.81)</td>
<td>36 (16.00)</td>
<td></td>
</tr>
<tr>
<td>Former</td>
<td>101 (46.98)</td>
<td>107 (47.56)</td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>80 (37.21)</td>
<td>82 (36.44)</td>
<td>0.99</td>
</tr>
<tr>
<td>Years smoked, mean (SD)</td>
<td>36.29 (14.74)</td>
<td>35.90 (14.48)</td>
<td>0.85</td>
</tr>
<tr>
<td>Number of cigarettes/day, mean (SD)</td>
<td>26.88 (17.42)</td>
<td>24.89 (15.61)</td>
<td>0.40</td>
</tr>
<tr>
<td>Pack-year, mean (SD)</td>
<td>50.05 (39.66)</td>
<td>46.42 (32.77)</td>
<td>0.48</td>
</tr>
<tr>
<td>Alcohol consumption</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>56 (26.05)</td>
<td>49 (21.78)</td>
<td></td>
</tr>
<tr>
<td>Previously consumed</td>
<td>62 (28.84)</td>
<td>74 (32.89)</td>
<td></td>
</tr>
<tr>
<td>Continues to consume</td>
<td>97 (45.12)</td>
<td>102 (45.33)</td>
<td>0.49</td>
</tr>
<tr>
<td>Tumor site</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Larynx</td>
<td>122 (56.74)</td>
<td>135 (60.00)</td>
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</tr>
<tr>
<td>Oral</td>
<td>68 (31.63)</td>
<td>59 (26.22)</td>
<td></td>
</tr>
<tr>
<td>Pharynx</td>
<td>25 (11.63)</td>
<td>31 (13.78)</td>
<td>0.42</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>I</td>
<td>142 (66.05)</td>
<td>138 (61.33)</td>
<td></td>
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<tr>
<td>II</td>
<td>73 (33.95)</td>
<td>87 (38.67)</td>
<td>0.30</td>
</tr>
<tr>
<td>Surgery</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>130 (61.03)</td>
<td>152 (67.56)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>83 (38.97)</td>
<td>73 (32.44)</td>
<td>0.15</td>
</tr>
<tr>
<td>Radiotherapy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>54 (25.23)</td>
<td>46 (20.44)</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>160 (74.77)</td>
<td>179 (79.56)</td>
<td>0.23</td>
</tr>
<tr>
<td>SPT/Recurrence</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>76 (35.35)</td>
<td>71 (31.56)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>139 (64.65)</td>
<td>154 (68.44)</td>
<td>0.40</td>
</tr>
</tbody>
</table>
Table 2. Effect of common genotypes of selected SNPs in placebo group and 13-cRA–treatment effect in patients with the common genotypes of these SNPs

<table>
<thead>
<tr>
<th>SNP</th>
<th>Gene</th>
<th>Gene function</th>
<th>Common genotype effect in placebo group</th>
<th>13-cRA–treatment effect in the common genotype group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Model&lt;sup&gt;a&lt;/sup&gt;</td>
<td>HR&lt;sup&gt;b&lt;/sup&gt; (95% CI)</td>
</tr>
<tr>
<td>rs3118570</td>
<td>RXRA</td>
<td>Retinoid X receptor</td>
<td>Dominant</td>
<td>3.33 (1.67–6.67)</td>
</tr>
<tr>
<td>rs1887427</td>
<td>JAK2</td>
<td>JAK/STAT signaling</td>
<td>Dominant</td>
<td>2.33 (1.41–3.85)</td>
</tr>
<tr>
<td>rs3025090</td>
<td>MMP3</td>
<td>Matrix metallopeptidase</td>
<td>Dominant</td>
<td>3.57 (1.64–7.69)</td>
</tr>
<tr>
<td>rs12137934</td>
<td>RAD54L</td>
<td>DNA repair</td>
<td>Additive</td>
<td>1.85 (1.23–2.78)</td>
</tr>
<tr>
<td>rs6596428</td>
<td>CDC25C</td>
<td>Cell division</td>
<td>Dominant</td>
<td>2.08 (1.28–3.45)</td>
</tr>
<tr>
<td>rs11244664</td>
<td>BCCIP</td>
<td>DNA repair, cell cycle regulation</td>
<td>Additive</td>
<td>1.67 (1.16–2.38)</td>
</tr>
<tr>
<td>rs2233913</td>
<td>SLC31A1</td>
<td>Membrane transporter</td>
<td>Dominant</td>
<td>3.03 (1.39–6.67)</td>
</tr>
<tr>
<td>rs11101992</td>
<td>GSTM5</td>
<td>Phase II metabolizing enzyme</td>
<td>Dominant</td>
<td>2.00 (1.23–3.33)</td>
</tr>
<tr>
<td>rs7040593</td>
<td>TSC1</td>
<td>AKT/mTOR signaling</td>
<td>Dominant</td>
<td>3.03 (1.37–6.67)</td>
</tr>
<tr>
<td>rs2274471</td>
<td>JAK2</td>
<td>JAK/STAT signaling</td>
<td>Additive</td>
<td>1.79 (1.18–2.70)</td>
</tr>
<tr>
<td>rs1410280</td>
<td>CDK8</td>
<td>Cell cycle progression</td>
<td>Dominant</td>
<td>4.35 (1.49–12.50)</td>
</tr>
<tr>
<td>rs3827665</td>
<td>TSC1</td>
<td>AKT/mTOR signaling</td>
<td>Dominant</td>
<td>3.57 (1.41–9.09)</td>
</tr>
<tr>
<td>rs9551427</td>
<td>FLT3</td>
<td>Kinase involved in regulation of hematopoiesis</td>
<td>Dominant</td>
<td>1.92 (1.19–3.13)</td>
</tr>
<tr>
<td>rs1243872</td>
<td>CA9</td>
<td>Tumor-associated carbonic anhydrase</td>
<td>Dominant</td>
<td>1.92 (1.19–3.13)</td>
</tr>
<tr>
<td>rs11602501</td>
<td>TNKS1BP1</td>
<td>Telomeric poly(ADP-ribose) polymerase</td>
<td>Dominant</td>
<td>1.96 (1.19–3.23)</td>
</tr>
<tr>
<td>rs739442</td>
<td>TSC1</td>
<td>AKT/mTOR signaling</td>
<td>Additive</td>
<td>1.56 (1.12–2.17)</td>
</tr>
<tr>
<td>rs10901431</td>
<td>MMP3</td>
<td>Matrix metallopeptidase</td>
<td>Dominant</td>
<td>1.96 (1.19–3.33)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Genetic model of inheritance

<sup>b</sup>Adjusted for age, gender, ethnicity, smoking status, tumor site, and tumor stage.

NOTE: Underlined P values remained significant following bootstrap resampling.

Figure 1. Kaplan–Meier event-free survival plot. A, effect of RXRA: rs3118570 genotypes on event-free survival stratified by genotype in placebo group. B, effect of 13-cRA treatment in patients with common genotype. C, effect of 13-cRA treatment in patients with variant-containing genotype. MST, median event-free survival time in months; *HR, adjusted for age, gender, ethnicity, smoking status, tumor site, and tumor stage.
these variants were associated with response to 13-cRA chemoprevention they would be poor prognostic markers to identify patients at risk of developing SPT/recurrence who would be candidates for chemoprevention.

**Efficacy of 13-cRA chemoprevention**

We analyzed the 13-cRA–treatment effect in patients having the common genotypes for each of the 45 loci that were identified in the placebo group as conferring an increased risk of SPT/recurrence. Of these 45 loci, 17 were associated with a 13-cRA–associated decreased risk of SPT/recurrence, compared with placebo. Of the 17 loci predicted a beneficial effect of low-dose 13-cRA compared with placebo, 13 were significant at \( P < 0.05 \) (Table 2), indicating that these 13 loci not only identified risk but sensitivity to 13-cRA chemoprevention as well. We also conducted bootstrap analysis for internal validation among SNPs significantly associated with increased in risk in the placebo group. All 4 of the genotypes that were not significant for risk following 13-cRA chemoprevention were those that did not remain significant following bootstrap analysis in the placebo arm for identification of prognostic risk factors.

A large majority (71.1%; \( n = 160 \)) of the patients receiving 13-cRA carried the common genotype for RXRA:rs3118570, and had a 38% reduction in risk of SPT/recurrence (95% CI, 0.43–0.90) with a corresponding significant event-free survival advantage of nearly 18 months from 75.1 to more than 93.0 months (\( P = 0.012 \)) compared with individuals receiving the placebo (Fig. 1B). In contrast, the minority of patients with at least one variant RXRA:rs3118570 allele would not receive benefit from 13-cRA and its use in this population would be contraindicated (Fig. 1C). Similar effects were observed for JAK2:rs1887427 and CDC25C:rs6596428, indicating that these candidate markers for poor prognosis also predict response to 13-cRA.

**Survival tree analysis for risk of developing SPT/recurrence in the placebo arm**

We conducted survival tree analysis to identify possible higher-order interactions among 17 loci that not only altered SPT/recurrence risk, but also predicted a beneficial effect of 13-cRA chemoprevention. Although 13-cRA did not have a significantly protective effect in 4 out of the 17 loci, they were included as candidates for gene–gene interactions in the placebo arm. In total, 10 of the 17 loci were predicted to have gene–gene interactions. The initial split on the tree structure was RXRA:rs3118570 suggesting that this marker is responsible for the most variation in SPT/recurrence risk (Fig. 2A). The 2 subsequent splits were for JAK2:rs1887427 and CDC25C:rs6596428, suggesting that these 2 loci are also strong candidates for increased SPT/recurrence risk. Carriers of common genotypes for RXRA:rs3118570, CDC25C:rs6596428, BCCIP:rs11244664, and FLT3:rs9551427 had shortest event-free duration of only 3.7 months and were in the highest risk grouping (node 11, Fig. 2A). Interestingly, and as would be expected on the basis of the common genotypes increasing risk of SPT/recurrence, patients with both variant RXRA:rs3118570 and JAK2:rs1887427 alleles were in the lowest risk grouping, and had a median event-free survival time of 68.8 months (node 1, Fig. 2A). When nodes with similar risk profiles were grouped, a significant difference in SPT/recurrence risk among these groups was observed (Fig. 2B). This translated into a dramatic reduction in median event-free survival time of nearly 60 months (\( P = 3.35 \times 10^{-17} \)) to only 8.8 months for those in the highest risk group (group 4) compared with more than 93 months for those in the low-risk group (group 1; Fig. 2C). These results remained significant after bootstrap resampling, indicating that the groupings identified in the survival tree analysis are reliable indicators of SPT/recurrence risk.

**13-cRA efficacy by patients’ genetic combination**

Combinations of RXRA:rs3118570, JAK2:rs1887427, and CDC25C:rs6596428 were able to further enhance the separation of patients who would receive benefit from 13-cRA chemoprevention. In the study, 85 (37.8%) of the patients receiving 13-cRA carried both of the common genotypes for RXRA:rs3118570 and JAK2:rs1887427. These patients had a 65% reduction in risk of SPT/recurrence (95% CI, 0.20–0.60) and a significant increase in their event-free survival time by more than 33 months (\( P = 0.0001 \); Fig. 3A). Carrying all 3 of these common genotypes resulted in further separation of the patients who benefited from 13-cRA. For these individuals, chemoprevention intervention with 13-cRA was associated with a 76% reduction in SPT/recurrence (95% CI, 0.093–0.64). Although the size of this subgroup is smaller (\( n = 30 \)), it still comprised 13.3% of the population in the treatment arm and these patients had an event-free survival advantage of more than 61 months (\( P = 0.00034 \); Fig. 3B).

**SPT/recurrence risks stratified by site of development**

We analyzed the risk of SPT/recurrence stratified by site in the placebo and treatment arms. In the placebo group, patients with the common genotype for RXRA:rs3118570 had a 3.24-fold increased risk (95% CI, 1.46–7.19; \( P = 0.0039 \)) of developing an SPT/recurrence in the lung or head and neck. Carriers of the common genotype for the following other 2 candidate loci also had a significantly increased risk of SPTs/recurrence in the lung or head and neck: JAK2:rs1887427 (HR = 2.33; 95% CI, 1.32–4.13; \( P = 0.0037 \)) and CDC25C:rs6596428 (HR = 2.20; 95% CI, 1.22–3.98; \( P = 0.0089 \)). Common genotypes at these 3 loci also conferred an increased risk of SPTs/recurrence in non-lung or non-head and neck sites: RXRA:rs3118570 (HR = 5.38; 95% CI, 1.20–23.81; \( P = 0.028 \)), JAK2:rs1887427 (HR = 4.18; 95% CI, 1.35–13.0; \( P = 0.013 \)), and CDC25C:rs6596428 (HR = 2.92; 95% CI, 1.05–8.06; \( P = 0.039 \)). In the 13-cRA arm, 2 of these 3 candidate loci had a statistically nonsignificant trend (posttreatment) toward protecting against lung or head and neck SPTs/recurrence, as follows: RXRA:rs3118570 (HR = 0.78; 95% CI, 0.41–1.45), and JAK2:rs1887427 (HR = 0.65; 95% CI, 0.36–1.16); this HR for CDC25C:rs6596428 was 1.14 (95% CI, 0.62–2.05). All 3 loci had a protective (albeit
statistically nonsignificant) effect on SPTs/recurrence in non-lung or non-head and neck sites: RXRA:rs3118570 (HR = 0.46; 95% CI, 0.18–1.15), JAK2:rs1887427 (HR = 0.74; 95% CI, 0.31–1.79), and CDC25C:rs6596428 (HR = 0.49; 95% CI, 0.13–1.82).

**Discussion**

We utilized a comprehensive approach to identify genetic factors that influence the prognosis for early-stage HNSCC and that modulate the preventive effect of 13-cRA intervention. In this study, we first conducted stratified analysis to first identify prognostic loci for SPT/recurrence risk that represent the majority of the patients in the placebo group. We then examined whether these loci also identified individuals who received benefit from chemoprevention response to 13-cRA treatment. Thirteen common genotypes were identified that met the criteria in that the genetic loci had a strong association with risk, and were also markers for response to 13-cRA. Together, these results provide evidence that 13-cRA was highly effective in a substantial percentage of this patient population.

The most significant findings were for the common genotype RXRA:rs3118570, which is located within an
intron of the gene encoding RXRA. Because an increased risk of SPT/recurrence in the placebo arm was observed only in patients carrying this genotype, RXRA:rs3118570 identified a majority of patients (71%) who are at a high risk of SPT/recurrence and thus are good candidates for intervention. The reason for the increased-risk association with RXRA:rs3118570 (compared with patients with variant genotypes) in the placebo group is not clear because the SNP is located in an intron. RXRA:rs3118570 is located approximately 100 bp upstream of a RXRA coding exon, which would be in proximity to potential splicing regulatory regions and enhancer elements (26). In addition, the haplotype structure containing rs3118570 includes a large portion of the 3’-untranslated region of RXRA, including several SNPs within potential microRNA binding sites. However, the effect on expression or function of RXRA, if any, remains unknown.

Of interest, RXRA:rs3118570 was not only prognostic but also predictive of the benefit of 13-cRA treatment, indicating that this receptor is a target for chemoprevention. This predictive ability of RXRA:rs3118570 has strong biological plausibility. RXRA is one of the retinoid receptors and plays a role in the transcriptional activation of retinoid responsive genes. RXRA:rs3118570, or another genetic locus in linkage disequilibrium with rs3118570, may be required for proper RXRA function in response to 13-cRA, and thus result in a distinctive 13-cRA-mediated therapeutic effect. The transcriptional regulatory function of 13-cRA is much lower than its all-trans and 9-cis isomers, and it has been reported that 13-cRA may modulate cellular activities through isomerization to the transcriptionally more active ATRA and 9-cRA (17–19). Consistently, 9-cRA has up to a 40-fold higher potency in binding to the RXRA receptor than the ATRA (27). Therefore, it is reasonable to assume that the effects observed in this study may be due to the 9-cRA-induced RXRA activation, and 9-cRA and ATRA activation of RAR/RXR heterodimers resulting in enhanced transcription of downstream target genes. Furthermore, because it has been reported that treatment of HNSCC cell lines by retinoids increased the expression of RAR mRNAs but not RXR mRNAs, it follows that the RXRA allele identified in this study is more likely to influence the function than the expression of RXRA. However, Dahiya and colleagues (28) reported that treatment of the LNCaP prostate cancer cell line with 13-cRA led to significantly increased RXRA mRNA expression, suggesting the presence of a context-specific and cancer-specific regulation of RXRA mRNA levels by retinoids.

The finding that RXRA:rs3118570 is linked to both increased risk of SPT/recurrence in the placebo group and favorable response to treatment with 13-cRA may also be explained by its differential state of activation before and after retinoid treatment. We found previously that the levels of ATRA are low in oral premalignant lesions in vivo (29). Therefore, we surmise that the same situation may exist in the patients in our clinical trial. In the absence of its ligand 9-cRA, RXRA is expected to suppress the expression of various genes. In contrast, after 13-cRA treatment and RXRA activation, many of these genes would be expressed. An interesting analogy was reported in breast cancer. RXRA has been found to be overexpressed in ductal carcinoma in situ, and to increase risk of breast cancer by more than 8-fold (30). Others found that 9-cRA was effective in chemoprevention of breast cancer (31). Additional in vitro and in vivo experiments will be needed to provide a definitive clarification on the functions of RXRA and this genetic locus in terms of the effect of 13-cRA in the prevention of SPT/recurrence in HNSCC. Furthermore, RXRA:rs3118570 was not among the top 20 SNPs associated with SPT/recurrence risk in entire group of 440 patients (24). Because the common genotype of RXRA was prognostic for SPT/recurrence in the placebo group but predictive of preventive benefit in the 13-cRA group, the effects were nullified in the overall, unstratified population of 440 patients. Two other genetic loci were also found to have similar prognostic and predictive abilities: JAK2:rs1887427 and
CDC25C:rs6596428. JAK2 encodes Janus kinase 2, a protein tyrosine kinase that is a key component of JAK/STAT signaling. This pathway is responsible for cytokine signaling in inflammation and response to stress. Genetic variation in JAK2 has been shown to be involved in myeloproliferative neoplasms (32), and JAK2 null mice are embryonically lethal due to erythropoietic and cardiac deficiencies (33, 34). Because of the importance of the JAK/STAT pathway in cancer development, it has been suggested as a future candidate target for chemoprevention (35). CDC25C is phosphatase that serves as a regulator of G2/M transition in the cell cycle and mediates this checkpoint in response to DNA damage. Retinoid treatment has shown to repress the D-type cyclins (cyclin D1, D2, and D3) resulting in cell cycle arrest at the G1 checkpoint (36).

Our results suggest that retinoids may also alter other phases of the cell cycle to control cancer cell proliferation to reduce the development of SPT/recurrence.

The common genotypes for RXRA:rs3118570, JAK2: rs1887427, and CDC25C:rs6596428 showed prognostic and predictive abilities individually, but this effect was magnified in patients who carried multiple common genotypes. Interactions among retinoids, retinoid receptors, and the JAK/STAT signaling have been established. Si and Collins showed that JAK2 activation was an intermediate between IL-3 signaling and RAR transcriptional activity during hematopoiesis (37). A similar activation of JAK/STAT signaling was observed in breast cancer cells. In this system, treatment with ATRA resulted in increased STAT1 phosphorylation, having an anti-inflammatory effect (39). Although a direct relationship among 13-cRA, RXRA, and JAK2 has not been established experimentally, these previous reports suggest that interactions are highly likely, giving biological plausibility to the results in our gene–gene interaction analysis. Similarly, the function of CDC25C has not been investigated within the context of 13-cRA treatment and RXRA. Additional studies will be required to fully elucidate the molecular mechanisms responsible for the predicted gene–gene interactions.

This study had several strengths. First, the patient population was obtained from and was representative of the largest prospective randomized chemoprevention trial in early-stage HNSCC patients. Our present study population did not differ significantly in patient characteristics from the other patients in the parent trial except for a 6% increased percentage of Caucasians. This was the first study specifically designed to identify genetic markers of a high-risk population, and genetic markers that can predict which patients will benefit from 13-cRA chemoprevention. We conducted bootstrap resampling analysis for internal validation, which supported our findings. However, we cannot exclude the probability of false-positive findings; therefore, further work is needed to replicate our findings, including analysis of these genetic loci in an external population. This approach could be extended to related retinoid trials in lung and head and neck carcinogenesis (40, 41). A prospective clinical trial in which patients would be genotyped for these 3 genetic loci prior to receiving 13-cRA will be required to determine whether stratification can successfully identify patients at a high risk of SPT/recurrence, and who will respond to 13-cRA chemoprevention to advance these findings into clinical practice.

From a clinical standpoint, having the ability to screen for not only risk of SPT/recurrence, but also response to 13-cRA chemoprevention prior to intervention has the power to significantly impact the quality of life and long-term outcomes for HNSCC patients. High-dose 13-cRA treatment is accompanied by side effects which can be severe enough to result in discontinuation of therapy (21). Although low doses of 13-cRA are better tolerated, these adverse events would be balanced by the expected benefit of reducing SPT/recurrence in a high-risk population. Furthermore, our results show that 13-cRA results in an increased risk of SPT/recurrence in individuals with certain genotypes. These individuals could quickly be screened out prior to initiation of chemoprevention to advert exposure to an unnecessary and potentially harmful agent. This strategy of using genetic markers to identify patients at high risk and who would most benefit from chemoprevention intervention is not specific to 13-cRA, and could be applied to other agents.

In conclusion, we have identified germline molecular markers for SPT/recurrence risk in HNSCC patients. These markers not only identify a high-risk population with the greatest need for chemoprevention, but also predict a favorable response to 13-cRA chemoprevention. Our results also indicate that 13-cRA is a highly effective chemoprevention agent for a substantial percentage of the population, when an individual’s genetic background is considered. These findings support the need for stratification of the HNSCC patient population in future chemoprevention trials to target those who will derive the most benefit from intervention. The need for such trials to validate our present findings is underscored by the lack of any alternatives for reducing risk in these patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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