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A Preliminary Study on Racial Differences in *HMOX1*, *NFE2L2* and *TGF β 1* Gene Polymorphisms and Radiation Induced Late Normal Tissue Toxicity

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Abstract

Purpose—This study tests whether racial differences in genetic polymorphisms of four genes involved in wound repair and response to radiation can be used to predict the occurrence of normal tissue late effects of radiotherapy and indicate potential therapeutic targets.

Methods and Materials—This prospective study examines genetic polymorphisms that modulate the expression of four genes involved in inflammation and fibrosis and response to radiation (*HMOX1*, *NFE2L2*, *NOS3* and *TGF β 1*). DNA from blood samples of 179 patients (~80% breast and head and neck) collected at the time of diagnosis by their radiation oncologist as exhibiting late normal tissue toxicity was used for the analysis. Patient demographics were: 56% Caucasian, 43% African-American, 1% other. Allelic frequencies of the different polymorphisms of the participants were compared to those of the general American population stratified by race. Twenty-six additional patients treated with radiation, but without toxicity at 3 months or later post-therapy, were also analyzed.

Results—Increased frequency of a long GT repeat in the *HMOX1* promoter was associated with late effects in both African-American and Caucasian populations. The single nucleotide polymorphisms (SNP) rs1800469 in the *TGF β 1* promoter and the rs6721961 SNP in the *NFE2L2* promoter were also found to significantly associate with late effects in African-Americans but not Caucasians. A combined analysis of these polymorphisms revealed that >90% of African-

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American patients with late effects had at least one and 58% two or more of these minor alleles. No statistical significance was found relating the studied *NOS3* polymorphisms and normal tissue toxicity.

Conclusions—These results support a strong association between wound repair and late toxicities of radiation. The presence of these genetic risk factors can vary significantly among different ethnic groups, as demonstrated for some of the SNPs. Future studies should account for the possibility of such ethnic heterogeneity in the late toxicities of radiation.

BACKGROUND

Radiation therapy is used in the treatment of approximately 60% of all cancer patients. Improvements in treatment delivery by increasing the amount of radiation targeted to a tumor and minimizing normal tissue exposure has enhanced the therapeutic ratio. Nonetheless, the potential for normal tissue toxicity remains a factor limiting the amount of radiation given for tumor control. With increasing effectiveness of different treatment modalities, there is a growing number of patients who are cancer survivors and potentially at risk for the late effects of radiation. Thus, preventing and reducing normal tissue toxicities of radiation are increasingly important components in any treatment plan (1,2).

Normal tissue toxicity is roughly divided into two categories, early and late. Early toxicities, such as mucositis, occur within the first few weeks of treatment and, for the most part, are self-limited and resolve with appropriate medical care. Late toxicities, such as pneumonitis and lung fibrosis, occur months to years post treatment and may be progressive and irreversible. Although the detailed mechanisms underlying normal tissue toxicities vary from tissue to tissue, the central underlying mechanism appears to be abnormal wound healing(1-4). Normal wound repair involves a transient inflammatory phase of increased expression of pro-inflammatory cytokines and recruitment of pro-inflammatory cells occurs followed by neovascularization and reepithelization, including the expression of anti-inflammatory molecules such as Transforming Growth Factor- β (TGF β), recruitment of fibroblasts and deposition of collagen, and extracellular matrix formation for wound closure. When fully regenerated, remodeling occurs with apoptotic removal of fibroblasts to form an acellular scar. Radiation-induced normal tissue toxicity such as lung fibrosis is seen as a chronic inflammatory response leading to unbalanced overexpression of collagen and the extracellular matrix(2).

Interpatient variability in response to defined treatment protocols has led to the proposal that a genetic basis underlies the probability of developing radiation-induced late toxicity. This hypothesis is supported by evidence showing no intrapatient variability in severity when two different treated areas of the same patient are compared with respect to normal tissue toxicity(2). A genetic basis underlying radiation toxicity is also found in the association of rare genetic syndromes with heightened radiosensitivity, e.g. Fanconi's anemia (2). Because of these correlations and potential medical benefits for predictive assays, a new field of radiogenomics has developed to investigate potential links between normal tissue radiosensitivity and patient genetic profile. These studies have mostly focused on specific single nucleotide polymorphisms (SNPs) in genes thought to have a role in the pathogenesis of this injury, e.g. *TGF β 1*, but more recently genome wide association approaches are being

considered(5). Identifying genes whose expression levels contribute to late radiation-induced tissue toxicity may offer potential therapeutic targets.

Consistent with evidence for genetic variability in risk of radiation induced toxicity is the observation of apparent differences in risk for some late effects between African-Americans and Caucasians. For example, in an analysis of >40,000 breast cancer patients treated with radiotherapy, African-American women had a statistically significant increased chance of experiencing a myocardial infarction (OR=1.33) or myocardial infarction plus ischemia (OR=1.23) after radiotherapy compared to Caucasian women(6). No candidate gene(s) has been identified to explain this finding. The demographics of our patient population, however, allows us to test whether racial differences in gene polymorphism profiles require consideration in associating the polymorphisms with the late effects of radiation.

The four candidate genes studied were chosen because of their roles in wound repair and fibrosis and their responsiveness to radiation exposure. To our knowledge, potential racial variations in the frequency of these polymorphisms and the risk of radiation-induced fibrosis have not been previously analyzed. TGF β has been extensively studied in radiation biology because of its roles in fibrosis and inflammation (1,2,7). Heme oxygenase-1 (HO-1) expression is induced by radiation *in vitro*(8) and *in vivo* in the lungs of mice(9). HO-1 deficiencies in mice and humans enhance the inflammatory effects of mild oxidative stresses consistent with its role as an anti-oxidative and anti-inflammatory molecule (10-13). Several investigations have also demonstrated that CO, a product of HO-1 catalysis, is anti-fibrotic and that TGF β and HO-1 in general oppose each other's activities (11). Nuclear regulatory factor-2 (Nrf2), a transcription factor encoded by the *NFE2L2* gene, regulates expression of several anti-oxidant proteins with the antioxidant response element in their promoters including HO-1, glutathione transferases, and glutathione peroxidase. Ionizing radiation stimulates *NFE2L2* transcriptional activity and mice deficient in *NFE2L2* show reduced life spans after thoracic radiation (8,14). As with HO-1, TGF β interacts antagonistically with Nrf2 (15). We have also focused on nitric oxide synthase-3 (*NOS3*) based on an association of some *NOS3* SNPs with the late effects of radiation and the evidence that NO stimulates Nrf2 activity and activates latent TGF β (16-20).

METHODS AND MATERIALS

Patients were recruited to this IRB-approved prospective study after obtaining informed consent. One group of patients (n=179) treated with curative intent were enrolled at the time they were diagnosed by their radiation oncologist with late radiation induced normal tissue injury according to the RTOG/EORTC Late Radiation Morbidity Scoring Schema (21). At enrollment, 5ml of blood was drawn and fractionated into serum and buffy coats by centrifugation. A second group of patients (n=26) were enrolled prior to beginning radiation therapy with curative intent. These patients provided blood before treatment, the day after the first treatment, every 2 weeks during treatment, at 6 months after treatment and at any subsequent time point when in the opinion of their attending radiation oncologist they developed a late complication from radiation. None of these patients had late toxicity at the time of analysis, and serve as the irradiated controls. Genomic DNA was isolated from frozen buffy coats using the ABI Prism 6100 Nucleic Acid Prep Station.

The *NOS3* SNPs were genotyped using VIC® and FAM® labeled primers from Applied Biosystems in custom-made plates according to the manufacturer's instructions and amplified on a ABI 7500 Fast Real Time PCR machine. The *NFE2L2* promoter SNPs were studied as described (22). The HO-1 GT repeat domain was isolated by PCR using the sense primer, 5'-AGAGCCTGCAGCT... TCTCAGA-3' and for the antisense primer, 5'-[6-FAM]AGAGCC...GCAGCTTCTCAGA-3'. Primers for the *TGFβ1* SNP were: 5'-CAGACTCTAGAGACTGTCAG-3' (forward) and 5'-GTCCTCTTTCTCTGGTGAC-3' (reverse). PCR products were sent to SeqWright DNA Technology Services (Houston, TX) for capillary sequencing and validation by chromatography for *HMOX1* and Sanger sequencing for *TGFβ1* and *NFE2L2*.

Each polymorphism is summarized to the observed minor allele frequency in two ethnic groups. The general population allele frequencies are from the PubMed SNP database, a research thesis for *HMOX1*(23), and ref. (24) for the *NFE2L2* SNPs. Test of significance is done by a two sided chi-square test for the hypothesis of the equality of the observed allele frequency and the population allele frequency using continuity correction(25). Population frequency of co-occurrence of SNPs on different genes is assumed to be independent as haplotypic dependence is not expected to span multiple genes. In our sample we checked the subjects with minor alleles and compared that to our computed population proportion using a large sample chi squared test for equality of proportions. The same procedure is used to compare the minor allele frequency between high toxicity (severity =2,3) and low toxicity (severity =0,1) groups within each ethnicity. All analyses were done using the statistical computing software R v3.0.2.

RESULTS

Patient demographics are shown in Table 1. Of the 205 patients 43% were African-American and 56% Caucasian reflecting the patient population served by our institution. Approximately 80% of the patients were treated for breast or head and neck cancer. Severity scores of 0 (no late effects), 1, 2 and 3 (mildest to severe) were determined by the patients' radiation oncologist and distributed 10%, 49%, 36% and 5% respectively. Because of small numbers within each subgroup, patients were subdivided into Low (score of 0 and 1) and High severity (score of 2 and 3) to determine if the SNPs independently predict the severity of late effects.

The length of the GT repeat in the *HMOX1* promoter determines transcriptional robustness – the longer the repeat the less transcriptional activity under conditions of oxidative stress (26). In the present analysis, the short or S-allele was defined as GT repeats < 26, M-allele between 26 and 32 and the L-allele >32 repeats (10-23). The results in Table 2 for *HMOX1* demonstrate a strong association between the L allele and late toxicity in patients of both races relative to the general population stratified by race. With Caucasian patients, there was also a negative association between S allele frequency and late effects with a statistically significant reduced S allele frequency in these patients relative to the Caucasian population as a whole. Prior studies confirmed significant differences in the GT repeat frequencies between the two ethnic groups (23). When patients were stratified into Low and High

degrees of severity, the L, M or S alleles were not predictive of severity (Supplemental Tables 1A and 1B).

Presence of minor alleles in three SNPs (rs35652124, rs6706649, rs6721961) of the *NFE2L2* promoter results in reduced expression of Nrf2 (27). When stratified by race, only the minor allele at rs6721961 is associated with a significantly higher risk for developing late effects in African-Americans with a trend in Caucasians (Table 2). However, the 95% CI for the allele frequency of the SNP within each ethnicity has some overlap, suggesting the effect to be broader than just one ethnicity. *NFE2L2* SNPs were also analyzed in terms of severity of late effects. As shown in Supplemental Data Tables 1C and D, none of the *NFE2L2* SNPs have any statistically significant predictive value for severity of late effects although trends were observed for rs6721961 and rs6706649, respectively, for African-Americans and Caucasians.

An initial analysis of the *TGFβ1* promoter SNP rs1800469 revealed no predictive value when the total patient pool was analyzed in agreement with recent studies (28). When stratified by race a significant association for developing late toxicity was observed for African-Americans expressing the minor allele but not for Caucasians (Table 2). The *TGFβ1* rs1800469 SNP was, however, significantly predictive of late toxicity in Caucasian patients (Tables 3A,B). In Caucasians with high toxicity indices (2 and 3) the minor allele SNP frequency was 0.47 compared to 0.26 for patients with low measures of severity ($p = 0.01$).

The present study examined two NOS3 SNPs that previous studies suggested are involved in radiation-induced late effects (17,18). No significant association of either SNP with late effects was observed (Table 2).

Besides looking at individual SNPs in isolation, combinations of these SNPs as a genetic risk score were also evaluated (Table 4). Of the 71 evaluated African-American subjects 66 or 93% carry at least one minor allele among the genes showing an association for late effects. The probability of this occurring in the general population, assuming independent segregation with the SNPs being on different genes is $1-(1-0.16)*(1-0.02)*(1-0.23) = 0.37$. This is significantly lower than the rate of occurrence in our sample population of African-American patients that were evaluable ($p < 0.001$). In addition, 58% of these patients showed 2 or 3 of the minor alleles of *HMOX1*, *NFE2L2* and *TGFβ1*. It is important to note that 3 of the 6 African-American patients not characterized by these 3 minor alleles carried a minor allele at the other *NFE2L2* promoter sites that depress Nrf2 expression. If one includes these in the analysis, 95% of the African-American patients express at least one of the minor alleles potentially associated with the late effects of radiation.

For Caucasians, 87% have at least one of the minor alleles compared to 48% for the general population (Table 4). However, only 39% of these patients have 2 or more of the minor alleles.

Table 5 is the analysis for patients who do not show late effects and for which data from both African-Americans and Caucasians have been combined because of the fewer patient numbers in this category, e.g. only 4 African-Americans with enough usable sequence data. The SNP frequencies in this table do not differ from our Caucasian patients with toxicities

but are significantly different from the African-American patients with late effects (Table 4). One explanation is that there is predictive value for these SNPs when combined in the case of African-Americans but possibly not Caucasians.

DISCUSSION

To our knowledge, this is the first study describing a genetic explanation for possible differences in susceptibility to late radiation-induced normal tissue injury between ethnic groups. We focused on selected polymorphisms of 4 genes whose gene products interact to modulate wound repair and for which there is experimental and clinical data suggesting involvement in the development of late normal tissue toxicity. The results for *HMOX1* show that regardless of race, the GT repeat in the promoter is associated with development of late toxicity. In contrast, the *TGFβ1* SNP rs1800469 and the *NFE2L2* SNP rs6721961 are associated with increased risk for late toxicities in African Americans but not in Caucasians.

Numerous studies have demonstrated a critical role for HO-1 in fibrotic and chronic inflammatory diseases (10-13, 29,30). One mechanism is HO-1-synthesized CO dependent suppression of TGFβ1-stimulated collagen synthesis as shown in mouse and human fibroblasts(12). Studies with endothelium show that the length of the GT repeat in the *HMOX1* promoter modulates HO-1 expression levels, i.e., the longer the repeat the lower the expression. Endothelial cells expressing the shorter repeat (<26) are more resistant to oxidative stress and produce lower levels of pro-inflammatory cytokines. Clinical trials also suggest that the length of repeat is predictive of cardiovascular problems(10). However, like the *TGFβ1* SNP studies, the results are mixed and may reflect racial differences in the populations examined that were not considered in the analyses, e.g. differences in the GT repeat frequencies between Caucasians and African-Americans(23).

Radiation induces a temporally triphasic enhanced expression of HO-1 in the rat lung (9). HO-1 expression is regulated by the *NFE2L2* encoded transcription factor, Nrf2 (18). *NFE2L2* knockout mice are more sensitive to thoracic radiation than wild type mice (14) and Nrf2 expression is depressed by a minor allele at rs672961 in the *NFE2L2* promoter. For African-Americans, an increased minor allele frequency at rs672961 in the *NFE2L2* promoter shows an association with late injury after radiation therapy. The mechanistic significance of this observation and association of the *TGFβ1* rs1800469 SNP with respect to whether African-Americans have an increased probability for radiation induced late effects is unclear but given that they are functionally antagonistic their combination would be expected to promote toxicity.

The minor allele frequencies of these 2 SNPS are both lower in the African-American general population relative to the Caucasian general population. This suggests that other interacting genes may be important. Of interest are genes involved in the pathogenesis of systemic sclerosis or scleroderma, an autoimmune connective tissue disease characterized by micro-vascular injury, and overproduction of collagen and other extracellular matrix components. This disease is more than twice as prevalent in African Americans as in Caucasians (31,32). Ethnic differences in anti-fibrotic hepatocyte growth factor expression and c-MET growth factor receptor activity in lung fibroblasts have been measured and

decreased activities of both are characteristic of African American patients with systemic sclerosis(32). Other studies demonstrate that African American endothelial cells exhibit higher levels of oxidative stress possibly due to altered expression of NADPH oxidase subunits and this may contribute to elevated inflammation and poor wound repair (33).

The most extensively evaluated SNP in radiation toxicity studies is rs1800469 in the promoter for *TGF β 1*. Focus on this SNP originated in correlations found between elevated serum or tissue levels of TGF β and the development of different fibrotic diseases or radiation injury(4,7,34-39). Attempts to demonstrate an association between radiation-induced toxicity and rs1800469 produced mixed results (36-39). A meta analysis of 2782 European breast cancer patients in 11 cohorts failed to show a significant association between rs1800469 and late toxicity or fibrosis(39). However, the demographics of this study group is unlikely to reflect the makeup of the US population, thus the findings may not be applicable to a more ethnically diverse American group of patients.

Conclusions from this study are tempered by the fact that we used as controls a relatively small group of cancer patients receiving radiation who did not develop late toxicity and a race stratified general population. While most studies assign a minimum latency of 3 months to allow for resolution of acute toxicity before scoring late effects, there is no time period beyond which the risk of late toxicity from radiation falls to zero. Thus, using a control group exposed to radiotherapy risks including patients who may, with longer follow-up actually develop late injury and thus may be misclassified. For this reason, we also chose to compare the irradiated patients to the general population stratified by race as a second control group, as this group is less likely to harbor significant groups of potentially misclassified patients. In addition, this type of comparison may provide useful risk information in the setting in which non-cancer patients might be exposed to significant amounts of ionizing radiation, such as in the setting of a nuclear accident or radiologic terrorism.

However this comparison presents its own weakness, ie. the polymorphisms are potentially involved in carcinogenesis. For example, a possible role of the (GT) n polymorphism in carcinogenesis have been examined. Results are mixed and are difficult to compare with our patient population since with many of these studies are based on Asian populations (40-44). However, in one American study comparing 500 post-menopausal women with breast cancer to 500 without found that women with *HMOX1* genotypes (LS + MM + MS + SS) not including LL and LM (reference group) had a reduced breast cancer risk of borderline significance (0.78 versus 1), when age-adjusted or fully adjusted for race, age, BMI, family history of breast cancer, and numerous other factors (40). Enhanced HO-1 expression and an additional increased expression in response to therapies characterizes most tumors (45-46). The increased HO-1 expression is anti-apoptotic and promotes angiogenesis both of which promote tumor growth but also mitigate normal tissue injury (2).

The roles of the *NFE2L2* promoter SNPs and Nrf2 expression in carcinogenesis have also been examined. Regardless of the tumor type Nrf2 expression is elevated in head and neck, bladder, colorectal, and lung tumors (47-50). Furthermore, the minor allele of rs6721961 SNP has not been associated with bladder, lung, gastric or colorectal carcinogenesis (48).

Our results testing only four genes in wound repair indicate that this pathway may have significant impact in the development of radiation-induced late effects. Not all polymorphisms of these genes have been analyzed and thus other polymorphisms of these genes may impact susceptibility to late radiation toxicity. These findings also indicate that racial differences represent important risk factors for normal tissue injury from radiotherapy and should be considered in future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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SUMMARY

This study shows that polymorphisms of three genes involved in wound repair and response to radiation can potentially predict late normal tissue toxicity if racial differences in polymorphism frequencies are considered. Increased frequency of a long GT repeat in the *HMOX1* promoter was associated with late effects in both African-Americans and Caucasian patient populations. Single nucleotide polymorphisms in the *TGF β 1* and *NFE2L2* promoters were significantly associated with late effects in African-Americans but not Caucasians.

Table 1

Patient Demographics (n=205)

Characteristics	Number	%
Gender		
Female	94	46
Male	111	54
Race (self declared)		
African-American	87	43
Caucasian	114	56
Asian	4	1
Site		
Breast	66	32
Head and Neck	97	47
Lung	18	9
Prostate	14	7
Other	10	5
Smoking Status		
Past	90	44
Never	47	23
Yes	55	27
Unknown	13	6
Dose (Gy)		
50.0	40	20
50.0-69.9	84	41
70.0	61	39
Diabetes		
African-American	28 of 87	32
Caucasian	21 of 114	18
Asian	1 of 4	25
Severity Scores		
0	26	12
1	95	46
2	69	34
3	10	5
Unknown	5	3

Table 2

Gene Polymorphisms of *HO-1*, *NFE2L2*, *TGFβ1* and *NOS3* in Patients with Radiation-Induced Normal Tissue Late Toxicities

<i>HMOX-1</i>											
GT Repeat (L>33)	NE ^a	LL	ML	MM	SL	SM	SS	L Allele Freq.	95% CI	Pop L Allele Freq.	P-Value
African-American	12	6	17	17	8	9	4	0.30	0.23-0.39	0.16	0.00
Caucasian	28	2	6	17	2	38	4	.09	.05-0.15	0.03	0.00
GT Repeat (S<26)	NE ^a	LL	ML	MM	SL	SM	SS	S Allele Freq.	95% CI	Pop S Allele Freq.	P-Value
African-American	12	6	17	17	8	9	4	0.21	0.14-0.29	0.24	0.42
Caucasian	28	2	6	17	2	38	4	0.35	0.27-0.43	0.44	0.04
<i>NFE2L2</i>											
A>G -653 rs35652124	NE ^a	AA	AG	GG	Allele Freq.		95% CI		Pop. Allele Freq.		P-Value
African-American	8	41	21	3	0.21		0.14-0.29	0.19			0.69
Caucasian	14	44	33	6	0.27		0.21-0.35	0.32			0.21
G>A -651 rs6706649	NE ^a	GG	AG	AA	Allele Freq.		95% CI		Pop. Allele Freq.		P-Value
African-American	8	57	7	1	0.07		0.03-0.13	0.05			0.42
Caucasian	14	65	18	0	0.11		0.07-0.17	0.10			0.82
C>A -617 rs6721961	NE ^a	CC	AC	AA	Allele Freq.		95% CI		Pop. Allele Freq.		P-Value
African-American	8	52	11	2	0.12		0.07-0.19	0.02			0.00
Caucasian	14	62	17	4	0.15		0.10-0.22	0.11			0.12
<i>TGFβ1</i>											
C>T rs1800469	NE ^a	CC	CT	TT	Allele Freq.		95% CI		Pop. Allele Freq.		P-Value
African-American	19	13	32	9	0.46		0.37-0.56	0.23			0.00
Caucasian	34	27	25	11	0.37		0.29-0.46	0.40			0.60

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NOS3									
T>C rs2070744	NE ^a	TT	CT	CC	Allele Freq.	95% CI	Pop. Allele Freq.	P-Value	
African-American	6	41	20	2	0.19	0.13-0.27	0.18	0.73	
Caucasian	8	29	38	12	0.39	0.32-0.47	0.42	0.53	

G>T rs1799983	NE ^a	GG	GT	TT	
African-American	33	26	10	0	0.14 0.07-0.25 0.15 0.92
Caucasian	43	14	25	5	0.40 0.30-0.51 0.35 0.35

^aNE : not evaluable; 95% CI: 95% Confidence interval

Table 3AAnalysis of *TGF β 1* and Severity of Late Effects in African American Patients

C>T rs1800469	Major Allele	Het	Minor Allele	Minor Allele Freq	P value
High	6	6	5	0.47	0.81
Low	13	29	6	0.43	

Table 3BAnalysis of *TGF β 1* and Severity of Late Effects in Caucasian Patients

C>T rs1800469	Major Allele	Het	Minor Allele	Minor Allele Freq	P value
High	11	12	9	0.47	0.01
Low	25	17	3	0.26	

Table 4

Combined Minor Allele Frequency Analysis

African-American [*] # Minor Alleles	0	1	2	3 or 4
Patient Count [*]	5	25	19	22
%	7	35	27	31
Cumulative %	10	42	69	100

Caucasian [#] # Minor Alleles	0	1	2	3 or 4
Patient Count [*]	12	43	28	7
%	13	48	31	8
Cumulative %	14	62	93	100

^{*} There were 83 African-American patients with late effects of which 12 were excluded in this combined analysis because of incomplete SNP analysis.

[#] There were 92 Caucasian patients with late effects of which 5 were excluded in this combined analysis because of incomplete SNP analysis

Table 5

Combined Minor Allele Frequency Analysis for Patients without Late Effects

# Minor Alleles	0	1	2	3 or 4
Patient Count *	4	11	10	1
%	15	42	39	4
Cumulative %	10	57	96	100

* There were a total of 26 African-American and Caucasian patients who at six months or later did not show physician-defined late effects.