Single-nucleotide polymorphisms in the p53 pathway regulate fertility in humans

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Contributed by Arnold J. Levine, April 17, 2009 (sent for review March 17, 2009)

The tumor suppressor protein p53 plays an important role in maternal reproduction in mice through transcriptional regulation of leukemia inhibitory factor (LIF), a cytokine crucial for blastocyst implantation. To determine whether these observations could be extended to humans, a list of single-nucleotide polymorphisms (SNPs) in the p53 pathway that can modify the function of p53 was assembled and used to study their impact on human fertility. The p53 allele encoding proline at codon 72 (P72) was found to be significantly enriched over the allele encoding arginine (R72) among in vitro fertilization (IVF) patients. The P72 allele serves as a risk factor for implantation failure. LIF levels are significantly lower in cells with the P72 allele than in cells with the R72 allele, which may contribute to the decreased implantation and fertility associated with the P72 allele. Selected alleles in SNPs in LIF, Mdm2, Mdm4, and Hausp genes, each of which regulates p53 levels in cells, are also enriched in IVF patients. Interestingly, the role of these SNPs on fertility was much reduced or absent in patients older than 35 years of age, indicating that other functions may play a more important role in infertility in older women. The association of SNPs in the p53 pathway with human fertility suggests that p53 regulates the efficiency of human reproduction. These results also provide a plausible explanation for the evolutionary positive selection of some alleles in the p53 pathway and demonstrate the alleles in the p53 pathway as a good example of antagonistic pleiotropy.

LIF | implantation | selection | alleles

The tumor suppressor protein p53 plays a pivotal role in coordinating cellular responses to genotoxic stressors and in maintaining genomic stability (1). In response to stress, p53 activation leads to various cellular responses, including apoptosis, cell cycle arrest, or senescence. The p53 pathway is crucial for tumor prevention. In some circumstances, disruption of normal p53 function is a prerequisite for the development or progression of tumors. p53 is the most frequently mutated gene in human tumors; over 50% of tumors harbor mutations in the p53 gene (2).

Recently, a previously undescribed function of p53 in reproduction has been uncovered; p53 plays an important role in blastocyst implantation and maternal reproduction through regulation of leukemia inhibitory factor (LIF) in mice (3). LIF is one of the most important cytokines in implantation. In many mammalian species, including mouse and human, transiently increased expression of uterine LIF is coincident with the onset of implantation. $LIF^{-/-}$ mice have a defect in maternal reproduction attributable to the failure of implantation (4). $p53^{-/-}$ mice have impaired implantation because of decreased uterine LIF levels. Injection of exogenous LIF into $p53^{-/-}$ female mice can significantly enhance implantation and rescue impaired reproduction (3).

A significant proportion of human infertility remains unexplained, and inefficient implantation is thought to be an important cause of infertility (5). As in mice, LIF has been suggested to be an important factor for implantation in humans. LIF levels in the majority of women with unexplained infertility are significantly decreased, as measured in uterine flushing (6). The regulation of LIF and blastocyst implantation by p53 in mice suggests a potential role of p53 and its pathway in human fertility.

In humans, single-nucleotide polymorphisms (SNPs) have been identified in genes at critical nodes in the p53 pathways, including p53, Mdm2, Mdm4, and Hausp. Some SNPs, such as p53 codon 72 SNP and SNP309 of Mdm2, have been shown to modify the activity or the levels of the p53 protein and influence cancer susceptibility (7–9). Furthermore, recent studies of the haplotype structures of these SNPs in the p53, Mdm2, and Mdm4 genes in populations with different ethnic backgrounds suggest that these genes are under evolutionary selective pressures for certain alleles (10, 11). It is therefore possible that the genes undergoing selection in the p53 pathway might influence human fertility. p53 codon 72 SNP is a common coding polymorphism (12) which results in either an arginine (R72) or proline (P72) residue at codon 72. The p53 P72 allele is weaker than the R72 allele in inducing apoptosis and suppressing cellular transformation but appears to be better at initiating senescence and cell cycle arrest (7, 13). Notably, Coulam and colleagues (5) have demonstrated that P72 is enriched in patients from an in vitro fertilization (IVF) clinic with recurrent implantation failure. Mdm2 SNP309 (a T-to-G change in Mdm2 intronic promoter region) increases Mdm2 expression levels and leads to attenuation of p53 function (9). Recent studies on the haplotype SNP structures of Mdm4 and Hausp, 2 critical regulators of p53, indicate the presence of candidate SNPs that influence p53 function and subsequent cancer risk (11). Both genes have an allele that appears to be under positive evolutionary selection. Mdm4 is a structural homolog of Mdm2 and is a major inhibitor of p53 activity. HAUSP (herpesvirus-associated ubiquitin-specific protease) can stabilize Mdm2, Mdm4, and p53 as a specific deubiquitinase and is an important regulator of the p53 pathway (14).

To study the potential role of the p53 pathway in human fertility, these SNPs in the p53 pathway were assessed in a cohort of women with fertility problems in an IVF clinic along with a control group. We found that p53 codon 72 SNP had a significant impact on LIF expression levels, which could, in turn, result in different implantation rates and lead to different pregnancy outcomes in human populations. Indeed, we found that P72 was enriched in IVF patients and served as a risk factor for implantation failure and decreased pregnancy rates after IVF, especially in patients younger than 35 years of age. Selected alleles of

Author contributions: H.-J.K., Z.R., A.J.L., and W.H. designed research; H.-J.K., Z.F., Y.S., T.R.R., and W.H. performed research; M.E.M. contributed new reagents/analytic tools; H.-J.K., Z.F., A.G., T.R.R., Z.R., A.J.L., and W.H. analyzed data; and A.J.L. and W.H. wrote the paper.

The authors declare no conflict of interest.

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Table 1. Induction of p53 target genes in cells containing SNPs at p53 gene codon 72

	8 h after p53 activation		16 h after p53 activation	
	Proline	Arginine	Proline	Arginine
LIF	2.14 ± 0.26	4.24 ± 0.37	3.17 ± 0.35	6.94 ± 0.71
Puma	8.22 ± 1.51	17.22 ± 1.92	10.76 ± 0.95	18.19 ± 1.97
Gadd45	10.05 ± 1.63	18.1 ± 1.72	11.84 ± 1.02	18.51 ± 1.67
Fas	4.94 ± 0.38	6.5 ± 0.57	4.99 ± 0.43	6.11 ± 0.54
Cyclin G1	3.22 ± 0.45	3.8 ± 0.28	5.37 ± 0.61	5.87 ± 0.45
Apaf-1	2.92 ± 0.39	2.71 ± 0.32	2.33 ± 0.19	2.66 ± 0.22
p21	6.84 ± 1.51	5.75 ± 0.42	10.07 ± 0.89	9.86 ± 0.92

Soas-2 cells containing p53 P72 and R72 expression plasmids were cultured at 32°C for 8 h and 16 h to expression of active p53 protein. The expression levels of a panel of p53 target genes were determined by Taqman real-time PCR and normalized to the levels of β-actin. The induction fold of these genes was calculated at each time point.

SNPs in LIF, Mdm2, Mdm4, and Hausp genes were also enriched in IVF patients. The observation that these at-risk alleles of SNPs in the p53 pathway act predominantly on patients younger than 35 years of age suggests that the LIF levels play a major role in implantation failure in this group. In women older than the age of 35 years, other factors, such as aneuploidy, may predominate, because the statistical role of p53 and LIF becomes less noticeable in the cohort. These results clearly demonstrate the association of SNPs in the p53 pathway with human fertility and strongly suggest that p53 regulates human reproduction.

Results

The p53 Codon 72 SNP Has Differential Transcriptional Induction of LIF. It is well established that p53 codon 72 SNP has different transcriptional activity toward a subset of p53 target genes (7). To examine the impact of p53 codon 72 SNP on induction of LIF, human Saos2 (p53-null osteosarcoma) cells stably transfected with temperature-sensitive p53 P72 and R72 alleles (7), which express mutant p53 at 39 °C and WT p53 at 32 °C, were used. We analyzed the expression levels of several p53 target genes in this pair of isogenic cell lines following culture at 32 °C for 8 and 16 h by real-time PCR. As shown in Table 1, the R72 allele preferentially induces higher levels of transcription than the P72 allele for some p53 target genes, such as Puma and Gadd45, but these alleles show comparable transactivation (TA) of p21, cyclin G1, and Apaf-1. Notably, the regulation of LIF is significantly different in these cells; the LIF expression levels are about 2-fold higher in cells containing active p53 R72 protein compared with P72 protein. The regulation of LIF transcription by p53 codon 72 SNP was further analyzed in a pair of human melanoma cell lines containing WT p53 R72 (WM115) and P72 (WM278), using gamma-irradiation (IR) as a stress signal to activate endogenous p53. Cells were irradiated (5 Gy), and the expression levels of LIF were measured at 16 h after IR. As shown in Fig. 1, the induction of LIF was over 2-fold higher in cells with the R72 allele than in cells with the P72 allele. Because LIF is an important factor for implantation, the regulation of LIF expression by p53 codon 72 SNP suggests that the codon 72 SNP of p53 may affect implantation rate and fertility in humans.

The p53 P72 Allele Is Enriched in Young Patients with Infertility. To investigate the impact of p53 codon 72 SNP on human fertility, the frequency of these polymorphic variants was assessed in an IVF patient population recruited at Weill Cornell Medical College. A total of 272 women with unexplained infertility were included in this study. Of these, 166 patients are younger than 35 years of age and 106 patients are older than 35 years of age. Maternal age has a significant negative impact on fertility, and a major underlying cause seems to be chromosomal aneuploidy. To exclude the contribution of aneuploidy on infertility and to decrease pregnancy failure after IVF, patients older than 35 years of age included in this study underwent donor IVF procedures using oocytes from a donor of a younger age (15). Patients younger than 35 years of age underwent fresh IVF procedures using their own oocytes. Lymphoblastoid cell lines established from 100 healthy Caucasian individuals (Caucasian 100) and women recruited as controls for the Women's Insights and Shared Experiences (WISE) study were used as controls to determine the frequency of SNPs in a normal population. As shown in Table 2, the frequency of P72, which is weaker in the induction of LIF expression, is significantly enriched in the IVF patient group (33.1% vs. 22.7%; $P = 1.8E\text{-}06$). The enrichment is more significant in young patients (<35 years), who are more likely to have implantation problems compared with older patients, who also demonstrate enhanced aneuploidy. The same trend is observed in older patients (≥35 years), but it is less significant ($P = 0.03$). These results confirm those of Coulam and colleagues (5) and strongly indicate that P72 is involved in human fertility, especially at the implantation stage, which is consistent with our previous finding that p53 regulates implantation in mice.

The p53 P72 Allele Is Associated with Poor Implantation and Pregnancy Rates in Young Patients with Unexplained Infertility. The impact of p53 codon 72 SNP on implantation and pregnancy after IVF was investigated in patients with infertility. Implantation rate was calculated as gestational sacs per embryo transferred. A serum human chorionic gonadotropin (hCG) level >5 mIU/mL at 9–11 days after embryo transfer was defined as the establishment of pregnancy. Evidence of fetal cardiac activity by transvaginal ultrasound examination at 6–7 weeks of gestational age was defined as a clinical pregnancy.

In young patients under the age of 35 years, P72 appears to be

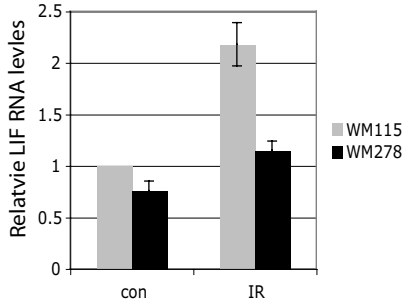

Fig. 1. The regulation of LIF expression levels by p53 with different SNPs at codon 72. Human melanoma cell lines homozygous for R72 (WM115, gray bar) and homozygous for P72 (WM278, black bar) were irradiated with 5 Gy of gamma-IR. The RNA expression levels of LIF were measured at 16 h after IR by real-time PCR. All values were normalized to the levels of β-actin, and the averages of 3 independent experiments are represented. con, control.

Table 2. Significant enrichment of selected alleles of genes in the p53 pathway in IVF patients

Gene	Genotype of SNP	Controls			IVF patients					
		Caucasian 100 *n* (%)	WISE control *n* (%)	Total *n* (%)	<35 years		≥ 35 years		Total	
					n (%)	*P**	*n* (%)	*P**	*n* (%)	*P**
p53 (rs1042522)	GG	57 (56.4)	653 (61.0)	710 (60.6)	68 (41.0)	2.8E-05†	53 (50.0)	0.13	121 (44.5)	2.1E-05†
	GC	35 (34.7)	357 (33.3)	392 (33.4)	79 (47.6)		43 (40.6)		122 (44.8)	
	CC	9 (8.9)	61 (5.7)	70 (6.0)	19 (11.4)		10 (9.4)		29 (10.7)	
	G	149 (73.8)	1663 (77.6)	1812 (77.3)	215 (64.8)	1.8E-06†	149 (70.3)	0.03†	364 (66.9)	1.8E-06†
	C	53 (26.2)	479 (22.4)	532 (22.7)	117 (35.2)		63 (29.7)		180 (33.1)	
LIF (rs929271)	TT	49 (49.5)	357 (54.2)	406 (53.6)	65 (39.1)	0.01†	50 (47.2)	0.48	115 (42.3)	0.02†
	GT	37 (37.4)	233 (35.4)	270 (35.6)	80 (48.2)		46 (43.4)		126 (46.3)	
	GG	13 (13.1)	69 (10.5)	82 (10.8)	21 (12.7)		10 (9.4)		31 (11.4)	
	T	135 (68.2)	947 (71.8)	1082 (71.4)	210 (63.3)	0.008†	146 (68.9)	0.43	356 (65.4)	0.02†
	G	63 (31.8)	371 (28.2)	434 (28.6)	122 (36.7)		66 (31.1)		188 (34.6)	
Mdm2 (rs2279744)	TT	42 (42.0)	492 (40.7)	534 (40.8)	48 (28.9)	0.05†	37 (34.9)	0.70	85 (31.2)	0.05[a]
	TG	45 (45.0)	526 (43.5)	571 (43.7)	88 (53.0)		49 (46.2)		138 (50.7)	
	GG	13 (13.0)	190 (15.7)	203 (15.5)	30 (18.1)		20 (18.9)		49 (18.0)	
	T	129 (64.5)	1510 (62.5)	1639 (62.7)	184 (55.7)	0.03†	123 (58.0)	0.35	308 (56.6)	0.03†
	G	71 (35.5)	906 (37.5)	977 (37.3)	148 (44.3)		89 (42.0)		236 (43.4)	
Mdm4 (rs1563828)	CC	46 (45.5)	560 (48.2)	606 (48.0)	62 (37.3)	0.07	42 (39.6)	0.01†	104 (38.2)	0.01†
	TC	47 (46.5)	468 (40.3)	515 (40.8)	81 (48.8)		41 (38.7)		122 (44.9)	
	TT	8 (8.0)	134 (11.5)	142 (11.2)	23 (13.8)		23 (21.7)		46 (16.9)	
	C	139 (68.8)	1588 (68.3)	1727 (68.4)	205 (61.7)	0.05†	125 (59.0)	0.02†	330 (60.7)	0.002†
	T	63 (31.2)	736 (31.7)	799 (31.6)	127 (38.3)		87 (41.0)		214 (39.3)	
Hausp (rs1529916)	GG	45 (45.0)	NA	45 (45.0)	61 (36.7)	0.003†	51 (40.2)	0.05†	112 (41.2)	0.006†
	GA	47 (47.0)	NA	47 (47.0)	64 (38.6)		36 (38.2)		100 (36.8)	
	AA	8 (8.0)	NA	8 (8.0)	41 (24.7)		19 (17.9)		60 (22.1)	
	G	137 (68.5)	NA	137 (68.5)	186 (56.0)	0.004†	138 (65.1)	0.46	324 (59.6)	0.03†
	A	63 (31.5)	NA	63 (31.5)	146 (44.0)		74 (34.9)		220 (40.4)	

*χ^2 test.
†Significant difference observed between IVF patients and controls.
NA, not analyzed.

a risk factor for implantation failure. The implantation rate is significantly lower in patients homozygous for P72 (19%) compared with patients carrying at least 1 allele of R72 (42%) ($P = 0.0028$), which, in turn, leads to a significantly lower clinical pregnancy rate in these patients homozygous for P72 (Fig. 2). In contrast, in older patients in whom chromosomal aneuploidy appears to be a major contributing factor to infertility, there is no significant difference on implantation and pregnancy rates among patients carrying different genotypes at p53 codon 72. The strong association of P72 with decreased fertility and implantation in young patients suggests that p53 regulates human fertility. This p53 polymorphism at codon 72 may account for an increased risk for infertility for a subset of young women because of lower LIF levels leading to impaired implantation.

A SNP in LIF Is Enriched in Young Patients with Infertility. The human LIF gene, which plays an essential role in implantation, has been recently identified as a p53 target gene (3). p53 regulates both basal and inducible transcription of LIF through direct sequence specific DNA binding and transcriptional activation. The importance of LIF variants in human fertility was investigated, and an association of a SNP in the 3′UTR of the LIF (rs929271) gene with human fertility was observed in this study. The G allele of this SNP is found at a rate of ≈30% in Caucasian Americans and does not exist in African Americans, which suggests that this SNP arose later in evolution after the migration out of Africa. As shown in Table 2, the G allele is significantly enriched in young patients under the age of 35 years (36.7% vs. 28.6%; $P = 0.008$) but not in older patients (31.1% vs. 28.6%; $P = 0.43$). Furthermore, in the control population of the WISE study, there is an association of the G allele with a history of fertility medication use [odds ratio (OR) = 2.5, 95% confidence interval (CI): 1.13–5.55] (Table 3), indicating the association of the G allele with infertility. These results demonstrate an association of a SNP in the LIF gene with infertility, especially in patients under the age of 35 years.

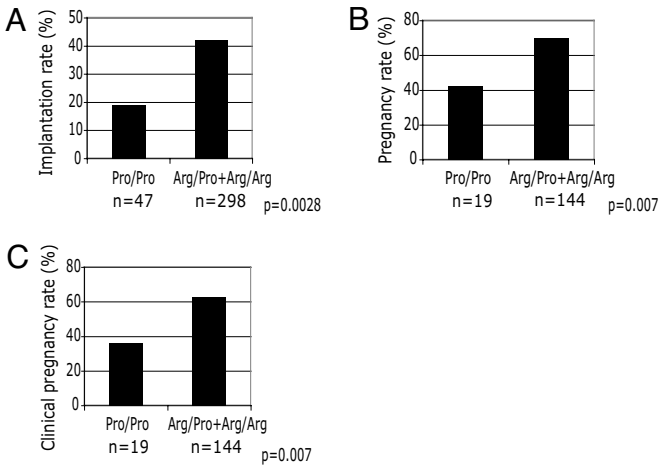

Fig. 2. Implantation rate and pregnancy rate after IVF in young patients with infertility carrying different genotypes at p53 codon 72. Implantation rate was calculated as gestations sacs/embryo transferred. A serum hCG level >5 mIU/mL at 9–11 days after embryo transfer was defined as a successful pregnancy. Clinical pregnancy rate is defined as fetal cardiac activity detected by transvaginal ultrasound at 6–7 weeks of gestation. p53 P72 was associated with a significantly lower implantation rate (*A*), pregnancy rate (*B*), and clinical pregnancy rate (*C*). Arg, arginine; Pro, proline.

Table 3. Adjusted ORs with 95% CIs for the effect of LIF genotype on reproductive characteristic in 679 WISE women

Genotype of SNP (rs929271)	Any use of fertility medications
T	(1)
G	2.50 (1.13–5.55)

SNPs of Genes in the p53 Pathway Are Associated with Infertility. To investigate the impact of the p53 pathway on human fertility further, SNPs in key regulators of the p53 pathway were genotyped in the IVF patient population. The SNPs examined include SNP309 in Mdm2, a SNP in the Mdm4 gene (rs2279744), and a SNP in the Hausp gene (rs1529916). All these SNPs appear to be under evolutionary selection pressures in the Caucasian population. For Mdm2 SNP309, the frequency of the G allele is ≈43% in Caucasian Americans and only 10% in African Americans. The study of the haplotype structures of the Mdm2 genes from African Americans, Caucasians, and Asians demonstrates that there are many different haplotypes for the T allele (it is older and has undergone mutations and recombinations many times), although there is only 1 G-allele haplotype found in Asian and Caucasian populations (arose more recently) (10). Similarly, the Mdm4 gene and the Hausp gene appear to have haplotypes in Caucasians under positive selection. In the Hausp gene, the frequency of the A allele is 33% in Caucasian Americans and only 16% in African Americans. Interestingly, there are many different haplotypes for the G allele, whereas there is only 1 A-allele haplotype found in Caucasian populations (arose more recently) (Fig. 3). For the Mdm4 gene, the frequency of the C allele is 67% in Caucasian Americans and only 30% in African Americans. There is a dominant C-allele haplotype in the Caucasian population and many different haplotypes for the T allele. These results suggest that the T allele is ancestral to the G allele in the Mdm2 gene, the G allele is ancestral to the A allele in the Hausp gene, and the T allele is ancestral to the C allele in the Mdm4 gene. Considering the recent establishment of the G-allele haplotype of Mdm2, the A-allele haplotype of Hausp, and the C-allele haplotype of Mdm4 in the Caucasian and Asian populations, along with their relatively high frequency in these populations, these observations suggest that these haplotypes are under positive selection in Caucasian and Asian populations and indicate the presence of candidate functional SNPs in these haplotypes that influence p53 function. These conclusions have been quantitatively verified using several approaches to determine the selection of genes in these populations (10, 11).

The evolutionary positive selection of these alleles in key nodes of the p53 pathway may have come about as a consequence of the need to keep the activity of the genes that regulate the p53 pathway at balanced and appropriate levels. The impact of p53 on reproduction is much more likely to be the most important selection pressure, because the impact of p53 in cancer risk and longevity occurs mainly in postreproductive years. To test this premise, we analyzed the frequency of SNPs of other genes in the p53 pathway in IVF patients. As shown in Table 2, there is clear

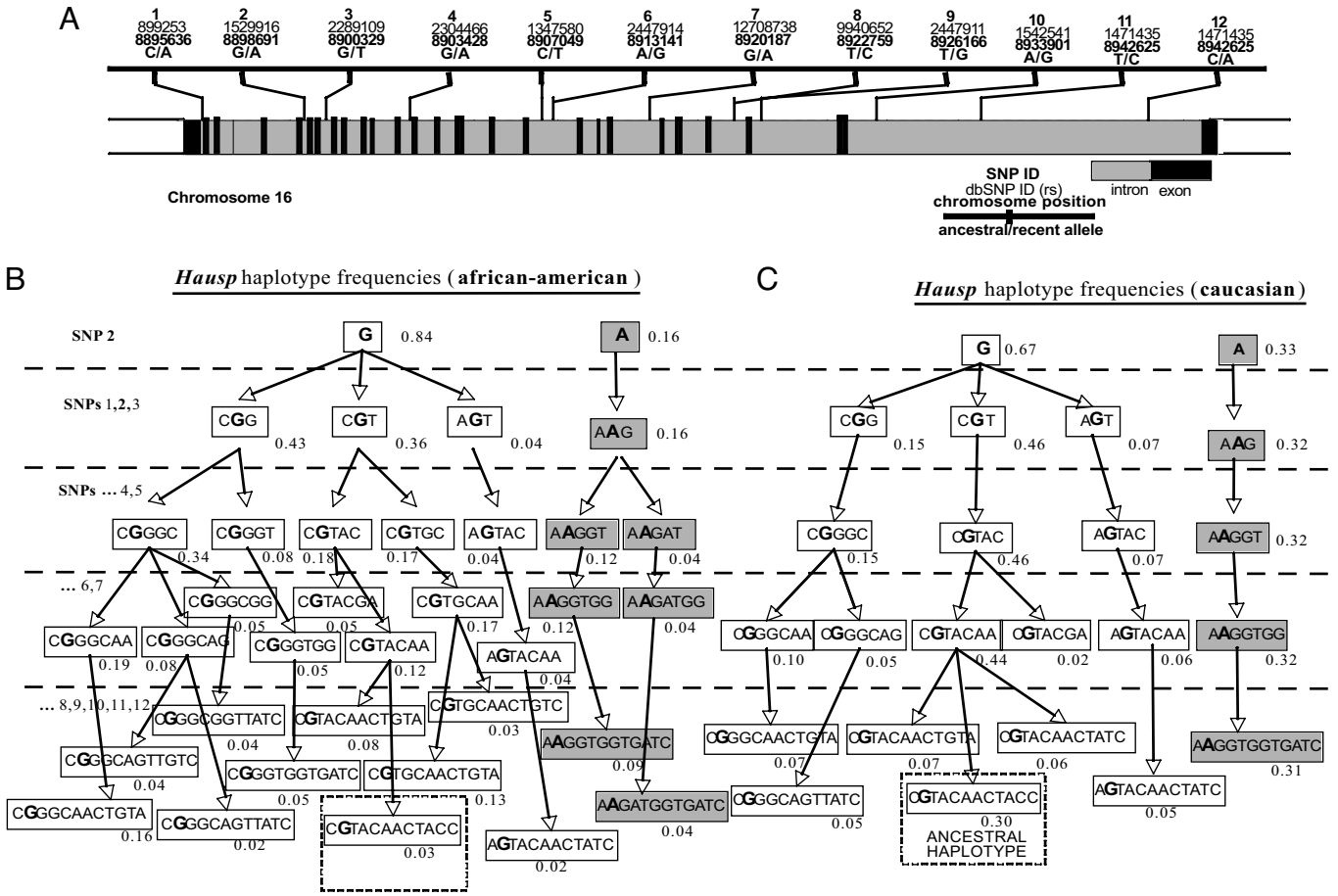

Fig. 3. Haplotype structure of the Hausp gene in African-American and Caucasian populations. (*A*) Schematic diagram of the Hausp gene and the SNPs genotyped for the study of haplotype structure. (*B* and *C*) Inferred haplotype frequencies in African-American and Caucasian populations, respectively. dbSNP, data base-SNP; ID, identification; rs, reference sequence.

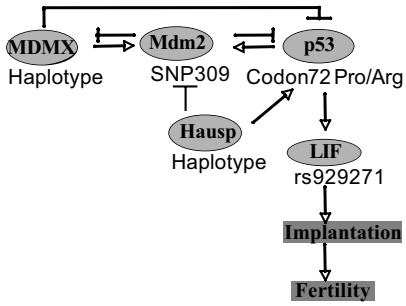

Fig. 4. SNPs in p53 and the p53 pathway associated with human fertility. Naturally occurring polymorphisms in the p53 pathway listed in the diagram, which modify the function of the p53 pathway, could have an impact on human fertility.

enrichment of selected SNPs in IVF patients. The significant enrichment of the Mdm2 SNP309 G allele and the A allele of Hausp gene was observed in young patients but not in older patients; this finding is similar to the observation made with the SNP in the LIF gene and p53 codon 72. These results demonstrate an association of the Mdm2 SNP309 G allele and Hausp A allele with infertility, especially in patients more likely to have implantation problems (under the age of 35 years), suggesting that these SNPs in Mdm2 and Hausp may have an impact on human fertility through attenuation of the p53 pathway. Interestingly, the enrichment of the Mdm4 T allele behaves as a significant variable in both the older patient group (41.0% vs. 31%; $P = 0.02$) and in young patients under the age of 35 years (38.3% vs. 31%; $P = 0.05$). This result suggests that Mdm4 may regulate fertility through p53-dependent and p53-independent pathways.

Discussion

The tumor suppressor p53 plays a crucial role in maintaining genomic stability and tumor prevention in somatic cells (1). Mdm2, Mdm4, and Hausp are all critical regulators of the p53 protein. Mdm2 binds to the p53 protein and degrades p53 through polyubiquitination of the p53 protein, sending it to the proteosome and blocking its ability to function as a transcription factor. Mdm4, a structural homolog of Mdm2, also binds to the amino terminus of p53 and is a key inhibitor of this protein. HAUSP is a critical component of the p53 pathway, acting as a specific deubiquitinase for p53, Mdm2, and Mdm4 (14). The Mdm2, Mdm4, and HAUSP proteins thus maintain p53 protein levels and activities, which are critical for an appropriate p53 transcriptional response to occur after a stress signal or in response to fertilization and implantation in the uterus (Fig. 4).

In humans, functional SNPs have been identified in both p53 and its negative regulator, Mdm2, which can alter the levels or function of p53. Interestingly, it appears that these SNPs (the p53 R72 allele and the G allele in Mdm2) are under evolutionary positive selection pressure in Caucasian and Asian populations (10, 11). Recent studies with the haplotypes of SNPs in the Mdm4 and Hausp genes also demonstrated the positive evolutionary selection toward selected alleles in these 2 genes in Caucasian populations. These observations suggested that p53 has evolutionarily conserved functions other than as a tumor suppressor. p53-like transcription factors are conserved from invertebrates to vertebrates, and the existence of p53-like proteins in short-lived organisms that do not exhibit adult cancer incidence, such as flies and worms, suggests that tumor suppression was not the original function for p53 and its pathway. Indeed, there is some evidence for p53 in reproduction and fecundity. In *Drosophila* and *Caenorhabditis elegans*, a p53-like protein is most commonly expressed in germ cells, and it functions in the surveillance of damaged DNA in germ cells to eliminate defective offspring from the population (16). In mice, p53 regulates blastocyst implantation through its target gene, LIF (3). This study demonstrates the association of SNPs in the p53 pathway with human fertility and strongly suggests that p53 regulates human fertility.

LIF is an important factor for implantation in humans. The LIF levels are significantly higher in cells with p53 R72 compared with isogenic cells with P72, a SNP with weaker p53 transcriptional activity. Therefore, altering the p53 activity by this SNP could result in different uterine LIF levels and implantation rates, which lead to different pregnancy outcomes in a human population. As shown in this report, p53 P72 is significantly enriched in IVF patients, especially in patients younger than 35 years of age. Furthermore, young patients homozygous for P72 have significantly lower implantation and pregnancy rates after IVF compared with patients carrying at least 1 allele of R72. Our results suggest that this p53 codon 72 polymorphism may increase the risk of implantation failure to a subset of young women under the age of 35 years undergoing IVF because of the lower LIF levels. Providing LIF to these patients at the implantation stage could potentially enhance their chance for pregnancy. For older patients in whom chromosomal aneuploidy and quality of the embryo seem to be major reasons for infertility, the enrichment of p53 P72 is less significant. These results strongly support the role of p53 in regulation of implantation and fertility in humans. Our observation is consistent with a recent report from Coulam and colleagues (5) showing that p53 P72 is associated with recurrent implantation failure in humans but is inconsistent with the result from another report that failed to observe such an association (17). However, the latter report used a cohort of patients who had an average age of 35–37 years and underwent fresh IVF procedure using their own oocytes. Therefore, the effect of aneuploidy on infertility from older women could be an important reason why the researchers failed to observe this association. Interestingly, the frequency of the p53 P72 allele (33%) in their cohort is very close to that in the patient cohort (33.1%) reported here, suggesting that there is potential enrichment of the p53 P72 allele in their cohort. They missed this observation because there was no control group included in their study. This may explain why no association of p53 codon 72 SNP with implantation and fertility was observed in their study, whereas in this study, each of 5 independent SNPs in different genes that interact epistatically within the p53 pathway all showed an association with human fertility, but only in women under the age of 35 years.

The Mdm2 SNP309 is a functional SNP that increases Mdm2 expression levels and attenuates the p53 pathway. SNPs in Mdm4 and Hausp genes can influence the functions of the p53 pathway, and the G allele of SNP 309 is associated with an increased cancer risk and a lower age of onset of cancers. There is a significant association of the Mdm2 SNP309 G allele and the A allele of Hausp gene with infertility in young patients but not in the older patients, which is similar to the observation made with p53 codon 72. These results suggest that Mdm2 and Hausp may be involved in the regulation of human fertility through p53 and its regulation of implantation. The association of the T allele of Mdm4 with infertility is observed in both young and older patient groups, suggesting that Mdm4 may regulate human fertility through p53-dependent and p53-independent pathways. Mdm4 interacts and inhibits not only p53 but other p53 family members, such as p63 and p73 (18). p63 plays an important role in DNA damage-induced apoptosis in primordial follicles in the female germ line (19). p73 encodes 2 major isoforms, TA p73, which contains the conserved TA domain and is highly homologous to the full-length of p53, and ΔNp73, which lacks the N-terminal TA domain. TA $p73^{-/-}$ mice also show a phenotype of infertility in female mice (20). Thus, it will be interesting to study the

potential role of p63 and p73 in human fertility in the future. The association of these SNPs with human infertility strongly indicates the involvement of the p53 pathway and family of transcription factors in fertility (Fig. 4). The minor alleles of all SNPs tested in this study are enriched in IVF patients, which demonstrates that the major allele of these SNPs contributes to improved fertility in Caucasian populations. This observation may explain the evolutionary positive selection of alleles of some SNPs in the p53 pathway. Obviously, these conclusions are not valid for African and African-American populations.

In summary, these data strongly suggest that the p53 pathway plays an important role in human fertility. Identifying higher risk polymorphisms for pregnancy failure could provide patients with more accurate predictions of their IVF success rates.

The functions of selected alleles in the p53 pathway genes explored here in fertility and in cancer demonstrate a cooperative pleiotropy; however, they demonstrate an antagonistic pleiotropy when the phenotypes of fertility and life span are compared. The functions of certain alleles being selected for in different genetic backgrounds (in Caucasian and Asian populations but not in African populations) emphasize the need for well-matched case-control populations. This also suggests that some of these same alleles could participate in hybrid dysgenesis leading to infertility as previously isolated groups begin to mate.

Materials and Methods

Cell Lines. Soas2-ts cells containing either P72 or R72 in the expression plasmid of p53 were established as previously described (7). Cells were maintained at 39 °C in DMEM supplemented with 10% vol/vol FBS and 400 μg/mL G418. Human melanoma cell lines WM115 carrying homozygous p53 R72 and WM278 cell lines carrying homozygous p53 P72 were derived from primary nonmetastatic tumors and were maintained in DMEM supplemented with 10% vol/vol FBS (7).

Study Participants.

1. Lymphoblastoid cell lines from the Coriell Diversity Cell Line panel. Lymphoblastoid cell lines (Caucasian 100) established from healthy Caucasian individuals (n = 100) were obtained from the Coriell Diversity Cell Line panel (Coriell).
2. The control group of the WISE study. The population of the WISE study has been described in detail previously (21). The present study involved 1,504 Caucasian female controls from the age of 50–79 years with no history of cancer at any site and having intact ovaries before menopause. In this population, 91% of women have ever been pregnant, which is very similar to the fertility frequency obtained from the U.S. National Survey of Family Growth, 1988–1995 (22).
3. Patients from an IVF clinic. A total of 272 women with unexplained infertility from the Center for Reproductive Medicine and Infertility at Weill Cornell Medical College were prospectively enrolled in this study. Of these, 106 patients were older than 35 years of age and 166 patients were younger than 35 years of age. Patients with severe male factor, poor ovarian reserve, or müllerian anomalies were excluded from the study. Patients underwent long (agonist) IVF protocols using luteal Lupron and gonadotropin stimulation in a step-down fashion.

Implantation rate was calculated as gestations sacs/embryo transferred. Pregnancy rate was defined as the detection of a serum hCG level >5 mIU/mL 9–11 days after embryo transfer. Clinical pregnancy rate was defined as the presence of fetal cardiac activity at 6–7 weeks of gestation.

Data collection was in accord with an Institutional Review Board-approved protocol.

Quantitative Real-Time PCR. Total RNA was prepared from cells using an RNeasy kit (Qiagen). Real-time PCR was performed in triplicate with Taqman PCR Mix (Applied Biosystems) in the 7000 ABI sequence Detection System (Applied Biosystems). All primers were purchased from Applied Biosystems. The expression of p53 target genes was normalized to β-actin gene.

Genotyping. SNPs genotyped in this study include p53 codon 72 SNP (rs1042522), Mdm2 SNP309 (rs2279744), SNP in LIF gene (rs929271), Mdm4 gene (rs1563828), and Hausp gene (rs1529916).

The status of these SNPs was determined in the WISE control group by using the ABI PRISM SNaPshot Multiplex Kit (Applied Biosystems) following the standard protocol as previously described (21). Genotypes were determined using GeneMapper 4.0 (Applied Biosystems).

The status of these SNPs was determined in the rest of the study participants by using a Taqman SNP genotyping assay. All primers were purchased from Applied Biosystems.

Data Collection and Statistical Methods. For the cohort of the WISE control population, a detailed questionnaire was used to assess prior medication use as well as exposures and reproductive history. OR estimates and 95% CIs were calculated to evaluate the relation between LIF polymorphism and prior use of fertility medications.

Multiple conditional logistic regression was used to account for age at the interview. Other confounder variables were considered, including reproductive history and exposure factors, such as exogenous hormone use. A variable was considered as a confounder if it changed the point estimate of any genotype effect by 10% or more.

Differences in genotype/allele distribution were evaluated using χ^2 analysis.

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