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NLRP7 and the Genetics of Postmolar Choriocarcinomas in Senegal

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Abbreviated title: NLRP7 in Senegalese Patients with Choriocarcinomas

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Running Title: Genetics of Choriocarcinomas in Senegal

Abstract

Gestational choriocarcinomas are malignant tumors of trophoblastic cells that affect 5 to 25% of women with sporadic hydatidiform moles depending on countries and studies. *NLRP7* is a major gene responsible for recurrent hydatidiform moles and recently mutations in this gene have also been shown in 13% women with sporadic, non-recurrent, moles. To investigate the role of *NLRP7* in the genetic susceptibility for the malignant degeneration of moles, we sequenced its 11 exons in 43 Senegalese patients with post molar choriocarcinomas. We report the presence of three novel *NLRP7* variants that were found only in patients but not in 100 controls from the Senegalese general population, 100 controls from the Tunisian general population, and 100 controls from the Canadian population. In addition, this analysis revealed significant differences in the frequencies of four non-synonymous *NLRP7* variants between European and Senegalese controls with the biggest difference being for variant G487E present at a minor allele frequency of 3.5% in Europeans, 18.1% in Tunisians, and 45.6% in Senegalese. Comparing human *NLRP7* and its paralog, *NLRP2*, with their mammalian counterparts revealed that allele E at position 487 is most likely the ancestral allele that was acquired in Africa but driven to low frequencies in Europeans and Asians due to migration, population bottlenecks, and selective pressures. This study is the first attempt to investigate the role of *NLRP7* in choriocarcinomas and highlights the higher frequencies of *NLRP7* variants in the general Senegalese and Tunisian populations both known to have higher frequencies of moles and choriocarcinomas.

Keywords: NLRP7, Choriocarcinomas, Mutation analysis, postmolar choriocarcinomas, Senegal.

Introduction

Hydatidiform mole (HM) is an aberrant human pregnancy with no embryo and excessive proliferation of trophoblastic cells. The common form of this condition is sporadic, not recurrent, and affects 1 in every 250 pregnancies in western countries (all types included) (Seckl *et al.*, 2010). This frequency is 2 to 10 times higher in developing and underdeveloped countries (Altieri *et al.*, 2003, Bracken, 1987, Grimes, 1984). At the histopathological level, HMs are divided into complete HMs (CHMs) and partial HMs (PHMs). Hydatidiform moles are also tumors of the trophoblast and are usually benign. However, in about 5% of CHM cases in western countries, moles degenerate into choriocarcinomas, which are malignant aggressive and highly metastatic tumors that might cause the death of the patients in cases of late presentation or inappropriate management. Again, in developing and underdeveloped countries, up to 30% of CHM degenerate into choriocarcinomas (Cisse *et al.*, 2004, Moodley *et al.*, 2003, Xue *et al.*, 2004) and in these countries, deaths from this condition affect up to 50% of choriocarcinoma cases (Moodley, et al., 2003, Cisse *et al.*, 2002, Eniola *et al.*, 2001, Khabouze *et al.*, 2002) Gestational choriocarcinomas may occur after any type of pregnancies, ectopic pregnancy, blighted ovum, spontaneous abortion, partial or complete hydatidiform mole, stillbirth, or normal pregnancy, but their risk increases by 1000-2000 times after a molar pregnancy (Altieri, et al., 2003, Buckely, 1996). We note that choriocarcinomas may also have a non-gestational origin resulting from male or female germ cell tumors. However, in this study, we deal only with gestational choriocarcinomas to which we refer hereafter, for simplicity, as choriocarcinomas.

Epidemiological studies have shown the following risk factors for choriocarcinomas, a history of a prior molar pregnancy, late maternal age, large parity (number of pregnancies), previous fetal wastage, oral contraceptive drugs, and maternal ethnicity. In studies performed in multiethnic

communities from Singapore, Hawaii, and the United States, Asian and African-American patients were found to have higher risks for choriocarcinomas than Caucasians living in the same communities (Buckely, 1996), which indicate the presence of genetic factors that are more frequent in Asians and African-Americans and that predispose the patients to choriocarcinomas.

NLRP7 (NOD-like receptor protein 7) is a gene that was originally reported as responsible for recurrent moles and associated reproductive wastage (Murdoch *et al.*, 2006). Later, mutations in this gene were also found in some women with one mole associated or not with other forms of reproductive wastage (Messaed *et al.*, 2011, Qian *et al.*, 2011). Most analyzed molar tissues from patients with *NLRP7* mutations have been found to be diploid biparental but some recently analyzed tissues were found to be diploid androgenetic or triploid dispermic indicating the involvement of *NLRP7* in different genotypic types of moles. To date, triploid dispermic and diploid androgenetic moles were all found in patients with one *NLRP7* defective allele while diploid biparental moles were found in patients with two defective alleles.

To investigate the role of *NLRP7* in the malignant degeneration of moles, we sequenced this gene in a cohort of 43 Senegalese women with postmolar choriocarcinomas. We report the identification of three novel *NLRP7* variants in the patients. However, we did not identify any significant association between non-synonymous variants (NSVs) in *NLRP7* and choriocarcinomas. In addition, we show increased frequencies of NSVs in controls from the general population of two African populations known to have higher incidences of moles and choriocarcinomas than Europeans.

Materials and Methods

Patients and control materials. All patients were recruited at the Obstetrics and Gynecology Department of the Aristide Le Dantec Hospital in Dakar (Senegal). The diagnosis of the moles was based on the hydropic degeneration of chorionic villi, the absence of fetal vessels, and the presence of important trophoblastic proliferation on most chorionic villi. No slides or tissues from the moles were available to us. All moles were histopathologically diagnosed as complete hydatidiform moles with the exception of one that was diagnosed as partial mole and had a triploid diandric genome. The diagnosis of choriocarcinomas was based on histopathological findings, absence of chorionic villi and presence of invasive proliferation of syncytiotrophoblasts and cytotrophoblasts surrounded by necrosis and hemorrhage. In about 25% of cases, choriocarcinoma tissues were available to us and their histopathological evaluations confirmed the previous diagnoses. Controls from three different ethnic groups were used in this study, 100 subjects from the Senegalese general population, 100 subjects from the Tunisian general population and 100 subjects from Canadian population of European ancestry. The full reproductive history of all the controls is not known, only the number of children of Europeans and Tunisians were available and the controls included males and females.

Mutation analysis. Mutation analysis was performed on blood DNA from the patients by genomic DNA amplification of the 11 exons of *NLRP7* (coding and 5' and 3' untranslated regions) and direct sequencing in the two directions. Sequences were aligned with reference sequence NM_001127255.1 and annotated according to the Human Genome Variation Society guidelines. Nucleotide numbering for mutations and variants uses cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence. Mutation analysis on control samples was performed under the same conditions used for patients by PCR amplification

of one amplicon, covering the region from amino acid 300 to amino acid 515, that were sequenced in one direction. In addition, the following variants, V319I, A481T, G487E, K511R, S675T, R721Q, R795C, A833T, and T1028A, were individually genotyped in 100 Senegalese controls using the Sequenom® iPLEX® Gold genotyping technology at the McGill University and Genome Quebec Innovation Centre.

Results and Discussion

Among the 43 unrelated Senegalese patients analyzed, we found three novel missense variants in *NLRP7,* in heterozygous state, that were not seen either in 100 Senegalese controls from the general population nor in another cohort of 100 African controls from the Tunisian general population (Tables I and II). In addition, none of these variants was seen in 100 Canadian controls of European ancestry from the general population (for additional controls see Supplementary Table SI). In one Senegalese patient, we found a heterozygous missense variant, R390H, which we previously reported in another patient originating from Guadeloupe and living in Canada who had had one complete mole, one normal pregnancy, and a choriocarcinoma after one of her 4 spontaneous abortions (Messaed, et al., 2011). This variant was also found in one Senegalese control whose reproductive history is not available. Using Polyphen 2 for human variations, a software that predicts possible impact of an amino acid substitution on the structure and function of a protein using structural, evolutionary, and comparative considerations, one missense variant, G498R, was predicted to be probably damaging, and two others, S675T and R721Q were predicted to be benign (Supplementary data Table SI). None of the three new variants is reported in the 1000 Genomes database in which data from more than 500 individual genotypes from various populations are available for *NLRP7*. These three missense variants could (i) be pathogenic, (ii) increase the susceptibility of the patients for moles or choriocarcinomas, or (iii) be simply very

rare variants that were not seen in our cohorts of controls. Future studies are needed to clarify their status with respect to moles and choriocacinomas. These data are in agreement with a previous study from our group showing *NLRP7* mutations in only 13% of patients with one mole and mainly in those who had had other forms of reproductive wastage in addition to their moles (Messaed, et al., 2011), which is not the case of these patients who did not have significant history of reproductive wastage. On the contrary, most of the patients included in this study are multiparous (and had had a large number of children), which is a known risk factor for choriocarcinomas in this population (Cisse, et al., 2002)

We note that we identified another variant, R795C, present at the same frequency in Senegalese patients and controls, but not in European controls, and that was predicted by Polyphen 2 to be possibly damaging. Beside R795C, we did not identify any other novel variant in the Senegalese controls that was not present in the patients.

Comparing the frequencies of NSVs between Senegalese patients and controls did not reveal any statistically significant difference indicating that the NSVs in *NLRP7* do not increase the genetic susceptibility for sporadic moles followed by choriocarcinomas in the analyzed patients (Table II). Our findings corroborate previous studies implicating environmental factors in the etiology of choriocarcinomas in Senegal (Cisse, et al., 2002). Although, we cannot exclude the involvement of other genes, the genetic predisposition for choriocacinomas, in general, does not seem to be strong because choriocarcinomas have never been seen or reported as a recurrent condition in the same patient or as a familial condition in related patients.

Another important observation in this study is the significant differences between the frequencies of four NSVs, V319I, A481T, G487E, and K511R between the Senegalese (Sub-Saharan African) and European (Northern) populations with the Tunisian population

(Mediterranean) having intermediate frequencies (Table II). Our allelic frequencies data are in agreement with data from the HapMap project on large cohorts from North American, Sub Saharan West African, and Asian populations (http://www.ncbi.nlm.nih.gov/projects/SNP/). Comparing human NLRP7 and its paralog, NLRP2, with several mammalian species revealed that two of these variants, Isoleucine at position 319 and Arginine at 511, are also present in other species (Figure I) and are probably very old. In addition, glutamic acid (E) at position 487 that was found at a MAF of 3.5% in Europeans versus 18.1% in Tunisians, and 45.5% in Senegalese is also present in all primates and lower mammalian species (Figure I). This minor allele, E, is present at low frequencies in Asian populations. Altogether, these data indicate that the rare allele in Europeans, E, is indeed the ancestral allele that was acquired in Africa but lost in Europeans due to migration, population bottlenecks, or/and the presence of positive and negative selective pressures. Recently, we found an association between rare *NLRP7* NSVs and recurrent reproductive wastage in Europeans (Messaed, et al., 2011). Despite the lack of association between these rare NSVs and choriocarcinomas in Senegalese patients, the higher frequencies of NSVs in African populations known to have higher frequencies of benign moles and choriocarcinomas is interesting and deserves to be re-investigated in the future in patients with recurrent reproductive wastage and larger cohorts of patients and controls.

Authors'roles

R.S. Designed the study, supervised the sequence analysis and mutation annotation, wrote the paper, and prepared the Tables and Figure. P.C. gathered the samples and recapitulated the clinical data. A-L.D. collected the samples and checked the diagnosis. W.C. performed sequencing and

analyzed the sequences. D.C. and A.G. provided DNA from Senegalese controls. S.A. provided DNA from Tunisian controls.

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Figure 1

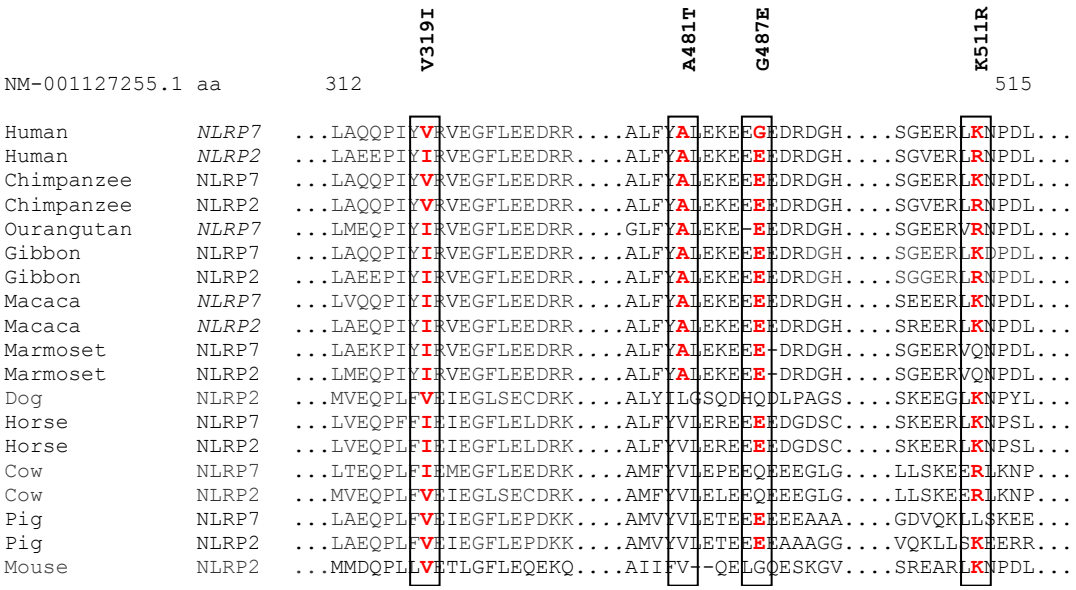
V319I
A481T
G487E
K511R
NM-001127255.1 aa 312 515
Human NLRP7 ...LAQQPIYVRVEGFLEEDRR....ALFYALEKEEGEDRDGH....SGEERLKNPDL...
Human NLRP2 ...LAEEPIYIRVEGFLEEDRR....ALFYALEKEEEEDRDGH....SGVERLRNPDL...
Chimpanzee NLRP7 ...LAQQPIYVRVEGFLEEDRR....ALFYALEKEEEEDRDGH....SGEERLKNPDL...
Chimpanzee NLRP2 ...LAQQPIYVRVEGFLEEDRR....ALFYALEKEEEEDRDGH....SGVERLRNPDL...
Ourangutan NLRP7 ...LMEQPIYIRVEGFLEEDRR....GLFYALEKE-EEDRDGH....SGEERVRNPDL...
Gibbon NLRP7 ...LAQQPIYIRVEGFLEEDRR....ALFYALEKEEEEDRDGH....SGEERLKDPDL...
Gibbon NLRP2 ...LAEEPIYIRVEGFLEEDRR....ALFYALEKEEEEDRDGH....SGGERLRNPDL...
Macaca NLRP7 ...LVQQPIYIRVEGFLEEDRR....ALFYALEKEEEEDRDGH....SEEERLKNPDL...
Macaca NLRP2 ...LAEQPIYIRVEGFLEEDRR....ALFYALEKEEEEDRDGH....SREERLKNPDL...
Marmoset NLRP7 ...LAEKPIYIRVEGFLEEDRR....ALFYALEKEEE-DRDGH....SGEERVQNPDL...
Marmoset NLRP2 ...LMEQPIYIRVEGFLEEDRR....ALFYALEKEEE-DRDGH....SGEERVQNPDL...
Dog NLRP2 ...MVEQPLFVEIEGLSECDRK....ALYILGSQDHQDLPAGS....SKEEGLKNPYL...
Horse NLRP7 ...LVEQPFFIEIEGFLELDRK....ALFYVLEREEEEDGDSC....SKEERLKNPSL...
Horse NLRP2 ...LVEQPLFIEIEGFLELDRK....ALFYVLEREEEEDGDSC....SKEERLKNPSL...
Cow NLRP7 ...LTEQPLFIEMEGFLEEDRK....AMFYVLEPEEQEEEGLG....LLSKEERLKNP...
Cow NLRP2 ...MVEQPLFVEIEGLSECDRK....AMFYVLELEEQEEEGLG....LLSKEERLKNP...
Pig NLRP7 ...LAEQPLFVEIEGFLEPDKK....AMVYVLETEEEEEEAAA....GDVQKLLSKEE...
Pig NLRP2 ...LAEQPLFVEIEGFLEPDKK....AMVYVLETEEEEAAAGG....VQKLLSKEERR...
Mouse NLRP2 ...MMDQPLLVETLGFLEQEKQ....AIIFV--QELGQESKGV....SREARLKNPDL...

Figure I. Multiple alignments of NLRP7 protein with its homologs and paralogs, NLRP2, in various mammalian species for three regions between amino acids 312 and 515. Multiple alignment was performed using blastp and Constraint-based Multiple Alignment Tool (COBALT) of the NCBI (http://www.ncbi.nlm.nih.gov/tools/cobalt/). Amino acid positions are according to reference sequence NM_001127255.1.

Table I. Summary of reprodutive outcomes and *NLRP7* mutation analysis in 43 Senegalese patients with postmolar choriocarcinomas

Case ID	Patient ID	Reproductive History	NLRP7 Mutation / Variant
MoSe122	M126	2 NP[a], 1 HM-CC[b]	p.[R390H(;)A481T(;)R795C]
MoSe123	M127	6 NP, 1 HM-CC	p.[G487E(;)**R721Q**]
MoSe124	M128	13 NP, 1 SA[c], 1 HM-IM-CC	p.[V319I; G487E];[V319I; G487E]
MoSe125	M132	3 NP, 3 SA, 1 HM-CC-death	p.[A481T];[=]
MoSe126	M133	10 NP, 1 HM-CC	p.[G487E];[=]
MoSe128	M136	9 NP, 1 SA, 1HM-CC	p.[R795C];[=]
MoSe129	M161	1 NP, 1 HM-CC-death	no NSVs
MoSe130	M163	7 NP, 1 HM-CC	p.[V319I(;)G487E(;)K511R]
MoSe131	M166	8 NP, 1 HM-CC	p.[V319I(;)A481T(;)G487E]
MoSe132	M167	2 NP, 1 HM-CC-death	p.[T1028A];[=]
MoSe133	M169	11 NP, HM-CC	p.[A481T];[A481T];[V319I(;)G487E(;)**G498R**(;)**A833T**]
MoSe134	M171	8 NP, HM-CC	p.[V319I(;)G487E(;)K511R]
MoSe135	M172	3 NP, 1 SB[d], HM-CC	p.[V319I(;)A481T(;)G487E]
MoSe136	M174	5 NP, 1 SA, 3 SB, HM-CC	p.[V319I(;)G487E(;)R795C]
MoSe137	M177	10 NP, 1 HM, CC	p.[T1028A];[=]
MoSe138	M182	9 NP, 1 HM-CC	p.[V319I(;)G487E(;)K511R]
MoSe139	M184	1 HM-CC	p.[V319I(;)G487E]
MoSe140	M188	1 HM-CC-death	p.[V319I(;)G487E]
MoSe141	M191	1 HM-CC	p.[V319I(;)A481T(;)G487E(;)T1028A]
MoSe142	M193	1 NP, 1 SB, 1 HM-CC	p.[V319I(;)A481T(;)G487E]
MoSe143	M194	8 NP, 1 SA, 1 HM-CC	p.[G487E];[G487E;T1028A]
MoSe144	M197	9 NP, 4 SA, 1 HM-CC	p.[A481T];[A481T];[V319I(;)G487E]
MoSe145	M199	8 NP, 1 HM-CC	p.[V319I;G487E];[V319I;G487E;T1028A]
MoSe146	M202	8 NP, 1 SA, 1 HM-CC	p.[V319I];[=]
MoSe147	M206	2 NP, 2 SA, 1 HM-CC	p.[V319I];[=]
MoSe148	M207	4 NP, 1 HM-CC	p.[V319I(;)A481T(;)G487E]
MoSe149	M225	12 NP, 2 SA, 1 HM-CC	p.[V319I];[V319I;K511R]
MoSe150	M226	4 NP, 1 SA, 1 HM-CC	p.[V319I;G487E];[V319I;G487E];[A481T(;)T1028A]
MoSe151	M227	6 NP, 1 HM-CC	p.[V319I(;)A481T(;)G487E]
MoSe152	M228	5 HM, 3 SA, 1 HM-CC	p.[V319I;G487E];[V319I;G487E];[A481T(;)T1028A]
MoSe153	M229	9 NP, 1 HM-CC M230	p.[**S675T**];[=]
MoSe154	M231	5 NP, 1 SA, 1 HM-CC	p.[V319I(;)A481T]
MoSe155	M233	6 NP, 1 HM-CC	p.[A481T];[=]
MoSe156	M236	11 NP, 1 HM-CC	p.[A481T];[=]
MoSe157	M237	5 NP, 2 SA, 1 HM-CC	p.[V319I(;)G487E(;)T1028A]
MoSe158	M238	12 NP, 1 SA, 1HM-CC	p.[V319I];[V319I];[G487E(;)T1028A]
MoSe159	M239	5 NP, 1 SA, 1 HM-CC	p.[V319I(;)G487E(;)T1028A]
MoSe160	M240	5 NP, 1 HM-CC	p.[V319I(;)G487E]
MoSe161	M204	1 HM-CC	no NSVs
MoSe162	M192	7 NP, 2 SA, 3 SB, 1 HM-CC	p.[V319I(;)A481T(;)G487E]
MoSe163	M179	1 HM-CC no other data	no NSVs
MoSe164	M180	1 HM-CC	no NSVs
MoSe165	M189	1 HM-CC	p.[V319I(;)G487E]

a, NP stands for normal pregnancy; b, HM-CC for hydatidiform mole followed by choriocarcinoma; c, SA for spontaneous abortion; SB, for stillbith. We note that the chronological order of the NP relative to the other forms of reproductive wastage are not known. However, all the indicated pregnancies occurred before the HM that lead to CC.

allele frequencies of non-synonymous *NLRP7* variants in Senegalese patients with as

Protein Variant	Minor allele	Senegalese Patients with HM-CC (n^a=43)	Senegalese Controls (n=100)	Tunisian Controls (n=100)	European Controls (n=100)
V319I	I	0.372	0.478	0.369	0.185
R390H	H	0.011	0.005	0	0
A481T	T	0.132	0.195	0.115	0.064
G487E	E	0.565	0.456	0.181	0.035
G498R	**R**	0.011	0	0	0
K511R	R	0.046	0.032	0.032	0.018
S675T	**T**	0.011	0	0	0
R721Q	**Q**	0.011	0	0	0
R795C	C	0.034	0.038	n.a.	0
A833T	**T**	0.011	0.005	n.a.	0
A1028T	T	0.104	0.130	n.a.	0

e number of subjects in each category. Variants seen only in patients are in bold character. inor allelic frequencies showing significant differences between populations.

Supplementary Table SI. Additional controls screened for the various novel variants identified in Senegalese patients and none had any of the four variants

New variant	Polyphen 2 scores for Human variations	Chinese general population (n=50)	Pakistani general population (n=38)	Total number of controls
c.1492G>A, p.G498R	0.983	50	38	88
c.2024G>C, p.S675T	0.025	50	38	88
c.2162G>A, p.R721Q	0.093		-	0
c.2487G>A, p.A833T	0.873	12	38	50