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Influence of *ESR1*gene Intronic Polymorphisms -397C/T (2234693) & -351A/G (9340799) in Etiopathogenesis of Reccurent Pregnancy Loss in Ethnic Kashmiri Women (North India)

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Abstract: ***Aim: Recurrent pregnancy loss is a multifactorial disorder involving environmental and genetic factors. The study is aimed to evaluate the association of two intronic polymorphisms of Estrogen receptor (ESR1) gene (-397C/T & -351A/G) with RPL in ethnic Kashmiri population. Methods: 180 patients with RPL and 200 control subjects were genotyped by a PCR-RFLP method. Results: Statistically significant increased frequencies of AG+GG genotype [OR, 3.61; 95% CI (2.30–5.67); P < 0.001] and the G allele [OR, 2.51; 95% CI (1.88–3.44); P < 0.001] of ESR1-351A/G (rs 9340799) is seen in RPL patients compared to healthy controls. Moreover, Haplotype analysis revealed that AG/CC, AG/CT, AG/TT, GG/CC, GG/CT and GG/TT genotype are significantly associated with RPL. Conclusion: In conclusion we found that the ESR1-351A/G SNP is significantly associated with the risk of RPL whereas the ESR1- 397C/T showed no association with RPL. Furthermore, various haplotype combinations (AG/CC, AG/CT, AG/TT, GG/CC, GG/CT and GG/TT) showed statistically significant association with RPL patients in ethnic Kashmiri population.***

I. INTRODUCTION

Pregnancy loss is the loss of the fetus before it has reached its viability. Recurrent pregnancy loss (RPL) is a disorder defined as repeated loss of 2 or more consecutive miscarriages before 22nd week of gestation and occurs in approximately 1% of fertile couples (27). Various high risk factors of RPL and possible causes include molecular and cytogenetic anomalies, anatomical disorders, immunological factors, hormonal abnormalities, environmental factors and thrombophilic defects (28, 29). More than 50% of cases of RPL have no anomaly and the specific cause remains unexplained (28). Estrogen is important steroid hormone secreted by ovary, influencing female reproduction through various aspects playing vital role during progression of pre-implantation, uteroplacental circulation, maintaining fetoplacental maturation and function (30). Estrogen binds to its receptors (ESR) 1 & 2 (members of nuclear receptor superfamily transcription factors) and exerts its effects by regulating the expression of multiple genes (14, 23). A study done on uterus of mice reported that the expression of *ESR1* was much higher than *ESR2* revealing the pivotal role of *ESR1* in RPL (8). Extremely polymorphic gene *ESR1* has been described and various studies reported that more than 2200 SNPs of *ESR1* gene have been identified (31). The *ESR1* gene is located on chromosome 6q25.1 with 8 exons and (-397C/T PvuII) & (-351A/G XbaI) located in intron 1 have been recognized as most widely studied SNPs associated with RPL (31, 32). These intronic variations do not lead to amino acid changes in protein & have been suggested as genetic markers for some *ESR1* related disorders due to linkage disequilibrium with different regulatory sequence variations that may influence expression and function of *ESR1* gene (33, 34). Various studies on association of ESR1 gene SNPs and RPL have shown different results based upon ethnicity and geographical distribution of the populations. Since, no study has been conducted as of now in our population which is ethnically conserved since consanguineous marriages are much more common, therefore in the present study, we evaluated the association of (−397C/T) & (−351A/G) polymorphisms of ESR1 gene with RPL in Kashmiri population.

II. MATERIALS & METHODS

A. Subjects

We enrolled in this case-control study a total 180 patients with at least two or more consecutive spontaneous abortions before 20th week of gestation referred from the Departments of Gynecology and Endocrinology, Sher-i-Kashmir institute of medical sciences. Subjects with any known causes of miscarriages including chromosomal abnormalities, immune factors and thrombophilic defects were excluded from the study.

The control population consisted of 200 healthy women classified according to ethnic origin with two or more successful pregnancies and no history of miscarriages, ectopic pregnancies and still births. Informed consent from all the subjects participating in our study was obtained. The study was approved by the Institutional Ethics committee of Sher-i-Kashmir institute of medical sciences.

B. Genomic DNA Analysis

For isolation of genomic DNA 4 milliliter of peripheral blood from each case and control subject was collected in EDTA vials. DNA was extracted by using a standard phenol/chloroform method. Genetic variants rs2234693 (C/T) and rs9340799 (A/G) polymorphisms in intron 1 of *ESR1* gene were detected by Polymerase chain reaction (PCR) followed by Restriction fragment length polymorphism (RFLP) analysis. Total volume of Polymerase chain reaction (PCR) was 25 µl containing 50 ng genomic DNA, 1× PCR buffer (Biotools, B & M Labs, S.A. Madrid-Spain) with 2mM MgCl2, 0.20mM dNTPs (Biotools, B & M Labs, S.A. Madrid-Spain), 0.4 µmol of each primer (Sigma-Aldrich Co. LLC·USA), and 1 U DNA polymerase (Biotools, B &M Labs, S.A. Madrid-Spain). PCR conditions were: initial denaturation step at 95 °C for 5 min, and 35 cycles each of denaturation for (95 °C for 30s), annealing (60°C for 30s) and extension (72 °C for 30s), followed by a final elongation at 72 °C for 5 min (Mahdavipour et al 2014 {8}, Anousha et al 2013 {26}). The primers used for amplification of *ESR1*-397C/T and *ESR1*-351A/G are given in Table 1. For RFLP analysis, 10 µl of PCR products were digested with the enzymes PvuII (-397C/T) and XbaI (-351A/G) (Fermentas Thermo Fisher Scientific Inc. Massachusetts, USA) (1 U at 37°C for 16 h), respectively. The restriction digested products and the PCR products (Table 1), were visualized through 3% agarose gel.

C. Statistical Analysis

The prevalence of allelic and genotypic frequencies of RPL cases and controls were compared using $\chi 2$ test. Fisher's exact test was used when $\chi 2$ test was violated (when one of the cell had an expected count of <1, or >20% of the cells had an expected count of<5). Odds ratios with 95% confidence intervals (CIs) were compared for the predisposition of the disease of specific alleles and genotypes. The results were considered statistically significant for ($P < 0.05$) and statistical analysis was done with SPSS v 20 and online software via http://vassarstats.net. Haplotype analysis was done manually.

III. RESULTS

A total of 380 individuals (180 RM cases and 200 healthy controls) were included in our study. The case group comprised of 180 women between 22 and 40 years of age. Mean age in patient group was ±30.01 years. No significant age-related differences were observed between the groups ($P > 0.05$) (Table 2).

The genotypic distributions of *ESR1*-397C/T P=0.07 and *ESR1*-351A/G P=0.069 polymorphisms in control population were in the Hardy-Weinberg equilibrium. Genotypes of −397 C/T (rs2234693) were defined by PCR-RFLP on basis of the presence of different patterns of bands as C/C (346 bp), C/T (103bp, 243 bp and 346 bp), and T/T (103 bp, 243 bp). Genotypes of −351A/G (rs9340799) were defined by the presence of two fragments of 148 bp and 198-bp (A/A), three fragments of 346bp, 148 bp and 198bp (A/G) , fragment of 346 bp (G/G).

In case of *ESR1*-397C/T the distribution of the genotypic and allelic frequency in cases and controls is given in Table 3. Among the patients with RM, frequency of *ESR1* -397C/T: CC, C T and TT genotypes was 60(33.3%), 94(52.22%) and 26 (14.45%) while in healthy controls it was 80 (40%), 102(51%) and 18 (09%) respectively.

The statistical analysis indicated the genotypic and allelic frequencies of *ESR1*-397 C/T polymorphism in cases and controls were not significantly different [OR, 1.2; 95% CI (0.8–1.9); P=0.3] and [OR, 1.29; 95% CI (0.96–1.74); P=0.09] respectively (Table 3). The association between ESR1α-397 C/T polymorphism and clinicopathological parameters didn't show any significant difference in the results (Table 4).

Among RM cases, frequency of *ESR1* −351 A/G: AA, AG and GG genotypes was 39 (21.67%), 105 (58.33%) and 36 (20.0%), while in the control population it was 100 (50.0%), 90 (45.0%) and 10 (5.0%). The statistical analysis indicated the genotypic and allelic frequencies of *ESR1*−351 A/G polymorphism in cases and controls to be significantly different [OR, 2.99; 95% CI (1.88–4.76); $P < 0.001$] [OR, 2.51; 95% CI (1.88–3.44); $P < 0.001$] (Table 3), indicating that the carriers of the G allele have a strong risk for the RM predisposition. Furthermore, the association between *ESR1* −351 A/G polymorphism and clinicopathological parameters was also carefully analysed (Table 4). TORCH is the only parameter that was significantly associated ($P < 0.05$) with the said polymorphism.

We observed strong linkage disequilibrium between the *ESR1*-397 C/T and *ESR1* −351A/G polymorphisms. Indeed, the two polymorphisms are only 50 base pairs apart in the first intron of the ESR1α gene. We performed the haplotype analysis of ESR1-397 C/T (*PvuII)* and *ESR1* −351A/G (*XbaI*) SNPs; the haplotype frequencies of ESR1-397 C/T (*PvuII)* and *ESR1* −351A/G (*XbaI*) SNPs are shown in Table 5. These common haplotypes ((p=**0.03**) (OR=2.57, 95% CI: 1.16-5.71) for AG/CC, (p=**0.04**) (OR=2.09, 95% CI: 1.04-4.2) for AG/CT, (p=**0.009**) (OR=4.71, 95% CI: 1.46-15.20) for AG/TT, (p=**0.0001**) (OR=7.71, 95% CI: 2.54-23.44) for GG/CC, (p=**<0.0001**) (OR=18, 95% CI: 3.61-89.61) for GG/CT, (p=**0.02**) for GG/TT.) detected a statistically significant association with the higher abortion risks (Table 5).

IV. DISCUSSION

Recurrent Pregnancy Loss is a multifactorial disorder, and various candidate genes have been evaluated by studying different polymorphic genetic markers. The phenotypic effects of polymorphisms are based on gene–gene interactions, genetic effects, and gene environment interactions (1). Genetic variations concerning thrombophilic and vascular genes are reported as a significant contributor to pregnancy complications (2), (3-5).Various Polymorphisms in the estrogen receptor genes could affect different estrogen dependent pathways which further influence vascular flow and tone, leading to the disruption in maintenance of pregnancy (6-8). *ESR1* plays an important role in the maintenance of uteroplacental and systemic circulation during pregnancy period (9). Reduced *ESR1* expression caused by a single nucleotide polymorphism of the *ESR1* gene has already been implicated in the pathophysiological aberriances of spontaneous abortions (10). To our knowledge this is the first study from our region on the association of *ESR1* polymorphism and RPL predisposition. We here investigated the association between two intronic polymorphisms of the estrogen receptor α gene (*ESR1* −397C/T: [rs2234693] and −351A/G:[rs9340799]), and RPL in Kashmiri Population (North India).

In our study we found statistically significant differences in the allelic and genotypic frequencies between cases and controls in case of *ESR1* −351A/G polymorphism [OR, 2.99; 95% CI (1.88–4.76); P < 0.001] [OR, 2.51; 95% CI (1.88–3.44); P < 0.001] (Table 3), respectively, indicating that presence of AG genotype or G allele instead of A allele imposes a significant risk for RPL predisposition. Our findings are similar with the studies of Pan *et al* 2014(11) in Chinese population and Pinedia *et al* 2010(12) in Spanish population. A Meta analysis done by Yin *et al* 2018 (13) revealed that rs9340799 (-351A/G) polymorphism is related to increased RPL risk in non-Asian population but decreased risk in Asian population. A possible reason for this phenomenon could be linkage disequilibrium in alleles which may differ in different ethnic populations and ESR polymorphisms in RPL may have opposite functions for Asians and non-Asians (13). However, a study from Guan *et al* 2002(14) did not find any association with the said polymorphism.

In case of *ESR1* −397C/T polymorphism a negative correlation was observed [OR-1.29; (0.96–1.74); P=0.09] with RPL in both case-control study and various clinicopathological parameters associated with RPL. Our study is consistent with the findings of Alessio *et al* (7) in brazillain population, Mahdavipour et al (8) in Iranian population and Hanna *et a*l (15) in Canadian population. However, a study in Spain found an association between *ESR1* −397C.T polymorphism and increase number of miscarriages (12). Both *ESR1* SNPs were observed as risk factors for various diseases such as Premature Ovarian Failure and Assisted Reproduction outcomes [cordts et al 2012(16) ; de Mattos et al 2014(17)].

Genetic haplotype is discerned as a combination of sets of alleles on the same chromosomal segment that imparts as block (18). As -351A/G and −397C/T are in strong linkage disequilibrium we combined the genotypes into haplotypes and various haplotype combinations AG/CC, AG/CT, AG/TT, GG/CC, GG/CT & GG/TT showed statistically significant differences between RPL cases and controls. Similar findings were reported by Pan *et al* 2014 (11) and Pinedia *et al* 2010 (12). Additionally, Molvarec et al 2006 concluded that TA haplotype of −397C/T and-351A/G ESR gene polymorphism is associated with increased risk of severe preeclampsia.

Disparate mechanisms have been known to exert phenotypic effects by intronic polymorphism. It may either augment or diminish the gene transcription, deliberately affecting the splicing of RNA, producing an alternative spliced mRNA variant with the remarkable changes in gene function (19–21). Moreover, intronic polymorphism may be linked to another truly functional sequence variant that ought to be genetic marker of another polymorphism. It is reported that *ESR1* −397 C allele contains part of a B-myb transcription factor binding site and functions as an intragenic enhancer (22). The transcription of myb is up-regulated by estrogen resulting in reduced expression of ESR1 gene in the presence of T allele, leading to a lower expression of an enzyme 17β-Hydroxysterioddehydrogenases(HSD), in estrogen synthesis pathway mediating the effects of estrogen which may get decreased leading to a relative estrogen deficit (23). The function of -351A/G polymorphism remains unascertained even though having functional implications.

ESR1 & 2 plays a pivotal role in maintenance of uteroplacental and systemic circulation during pregnancy (24). Decreased expression of *ESR1* gene has been implicated in pathological aberriances of spontaneous abortions (25).

Selection of few SNPs for ESR1 gene evaluated in this investigation deciphers the assessment of that particular site and the one's present in linkage disequilibrium with it. However, they do not elucidate all of the genetic variations in those genes. Therefore, the important functions of various rare mutations and SNPs towards the risk for RPL cannot be ignored. A Synergistic effect of combinations of SNPs especially in polymorphic genes is hard to appropriately describe in association studies. Functional explanations and studies and elaborative analysis of genetic variations within *ESR1* gene is extensively required and well designed studies with larger samples are further required to evaluate the association between *ESR1* polymorphism and Reccurent pregnancy losses.

V. CONCLUSION

In conclusion, AG+GG genotype of (-351A/G) polymorphism is a risk factor in predisposition towards RPL and the various haplotype combinations (AG/CC, AG/CT, AG/TT, GG/CC, GG/CT & GG/TT) for the SNPs are associated with the risk of Recurrent pregnancy losses whereas, other SNP (−397C/T) has no role in RPL in ethnic Kashmiri population.

Table1: Primer sequence, Annealing temperatures, Restriction Enzymes & lengths of digested fragments of ESR1- gene for polymorphic variants

Polymorphism	rs number	Primer Sequence	Restriction Enzyme	AT*	Amplicon size (bp)	RFLP pattern
ESR1α-397C/T	2234693	F: 5′- GATATCCAGGGTTATGTGGCA-3′£ R:5′-AGGTGTTGCCTATTATATTAACCTTGA-3′	PvuII	60ºC	346bp	CC-346bp CT-103bp,243bp,346bp TT-103bp,243bp
ESR1α-351A/G	9340799		XbaI			AA-148bp,198bp AG-346bp,148bp,198bp GG-346bp

AT*-Annealing Temperature

£-The same primer set was used to amplify for both polymorphisms variants

Table 2: Frequency distribution analysis of selected demographic and risk factors in Reccurent Miscarriage cases and controls

Variables	Cases N=180 (%)	Controls N=200 (%)	p- value
Age group ≥30 <30	 97 (53.89%) 83 (46.11%)	 105 (52.5%) 95 (47.5%)	0.8
Dwelling Rural Urban	 112 (62.22%) 68 (37.78%)	 123 (61.5%) 77 (38.5%)	0.91

Table 3: Genotypic frequencies of *ESR*-α- polymorphisms in Reccurent miscarriage cases and controls

Genotype	RM Cases n=180 (%)	Controls n=200 (%)	*OR (95% CI)*	*P-value*
ESR1α-397C/T				
CC	60(33.3)	80(40)	1.0(Reference)	
CT	94(52.22)	102(51)	1.2(0.8-1.9)	0.3
TT	26(14.45)	18(09)	1.92 (0.97-3.83)	
				0.08
Allele (2N)	360	400		
C	214 (59.44)	262(65.5)	Ref.	
T	146 (40.56)	138(34.5)	1.29(0.96-1.74)	0.09
ESR1α-351A/G				
AA	39(21.67)	100(50.0)	Ref.	P<0.001
AG	105(58.33)	90(45.0)	2.99(1.88-4.76)	
GG	36(20)	10(5.0)	9.2(4.18-20.39)	P<0.001
AG+GG	141(78.33)	100(50)	3.61(2.30-5.67)	P<0.001
Allele (2N)	360	400	Ref.	
A	183(50.83)	290(72.5)	2.5(1.88-3.44)	P<0.001
G	177(49.17)	110(27.5)		

Table 4: Association between *ESR1α-397C/T and ESR1α-351A/G* polymorphisms and various clinical and lab features of RM patients

Parameter	Cases (n=180)							
	ESR1α-397C/T				*ESR1α-351A/G*			
	CC(60)	*CT+TT(120)*	p-value	*OR*	*AA(39)*	*AG+GG(141)*	p-value	*OR*
Age								
≥ 30years(97)	33(34.02)	64(65.98)	0.87	0.93(0.5-1.74)	24(24.74)	73(75.26)	0.36	0.67(0.32-1.38)
< 30years(83)	27(32.53)	56(67.45)			15(18.07)	68(81.93)		
Dwelling								
Rural(112)	38(33.93)	74(66.07)	0.75	0.89(0.47-1.69)	26(23.21)	86(76.79)	0.58	0.78(0.37-1.65)
Urban(68)	22(32.35)	46(67.65)			13(19.12)	55(80.88)		
TORCH								
Positive(21)	06(28.57)	15(71.43)	0.8	1.28(0.47-3.50)	05(23.81)	16(76.19)	0	0.87(0.30-2.5)
Negative(159)	54(33.96)	105(66.04)			34(21.38)	125(78.62)		
APLA								
Positive(10)	03(30)	07(70)	1	1.17(0.29-4.72)	01(10)	09(90)	0.46	2.6(0.3-21)
Negative(170)	57(33.53)	113(66.47)			38(22.35)	132(77.65)		
ANA								0.88(0.27-2.89)
Positive(17)	07(41.18)	10(58.82)	0.59	0.68(0.25-1.9)	04(23.53)	13(76.47)	1	
Negative(163)	53(32.52)	110(67.48)			35(21.47)	128(78.53)		
AMH								
Normal(163)	54(33.13)	109(66.87)	Ref		36(22.09)	127(77.91)	Ref	
High(10)	04(40)	06(60)	0.73	1.34(0.36-4.97)	02(20)	08(80)	1	0.88(0.18-4.34)
Low(07)	02(28.57)	05(71.43)	1	0.81(0.15-4.30)	01(14.29)	06(85.71)	1	0.59(0.07-5.04)
APTT								
Normal(169)	54(31.95)	115(68.05)	Ref.		38(22.49)	131(77.51)	Ref	
High(10)	05(50)	05(50)	0.30	2.13(0.59-7.68)	01(10)	09(90)	0.46	0.38(0.05-3.2)
Low(01)	01(100)	0	0.32	-	0	01(100)	1	
FSH								
Normal(173)	58(33.53)	115(66.47)	1	0.79(0.14-4.2)	36(20.81)	137(79.19)	0.34	2.85(0.61-13.3)
Abnormal(07)	02(28.57)	05(71.43)			03(42.86)	04(57.14)		

LH Normal(180) Abnormal(0)	60(33.33)	120(66.67)	-	-	39(21.67)	141(78.33)	-	-
PRL Normal(180) Abnormal(0)	60(33.33)	120(66.67)	-	-	39(21.67)	141(78.33)	-	-
TESTOSTERONE Normal(169) Abnormal(11)	56(33.14) 04(36.36)	113(66.86) 07(63.64)	1	1.15(0.32-4.1)	39(23.08) 0	130(76.92) 11(100)	0.12	
TSH Normal(131) Hypothyroidism(47) Hyperthyroidism(02)	40(30.53) 20(42.56) 0	91(69.47) 27(57.44) 02(100)	Ref 0.15 0.58	1.68(0.84-3.35) -	31(23.66) 07(14.89) 01(50)	100(76.34) 40(85.11) 01(50)	Ref 0.22 0.42	0.56(0.23-1.39) 3.22(0.2-53)
CONSANGUINITY Yes(55) No(125)	19(34.55) 41(32.8)	36(65.45) 84(67.2)	0.86	0.92(0.47-1.80)	16(29.09) 23(18.4)	39(70.91) 102(81.6)	0.11	0.54(0.26-1.14)
PESTICIDE EXP Yes(17) No(163)	06(35.29) 54(33.13)	11(64.71) 109(66.87)	1	0.9(0.31-2.58)	06(35.29) 33(20.25)	11(64.71) 130(79.75)	0.21	0.46(0.16-1.35)
USG Normal(147) PCOS(33)	52(35.37) 08(24.24)	95(64.63) 25(75.76)	0.3	0.58(0.24-1.38)	31(21.09) 08(24.24)	116(78.91) 25(75.76)	0.8	1.19(0.49-2.91)
Family History Yes(27) No(153)	11(40.74) 49(32.03)	16(59.26) 104(67.97)	0.5	0.68(0.29-1.58)	06(22.22) 33(21.57)	21(77.78) 120(78.43)	1	0.96(0.35-2.57)

Table 5: Combined Haplotype Analysis of *ESR1α-397C/T and ESR1α-351A/G* polymorphisms in Reccurent Miscarriage cases and controls

Haplotype combination	Cases n=180 (%)	Controls n=200(%)	Odds Ratio	p-value
AA CC	14 (7.78)	36 (18)		
AA CT	17 (9.44)	28 (14)	1.56(0.65-3.7)	0.38
AA TT	08 (4.44)	12 (6)	1.71(0.59-5.08)	0.40
AG CC	30 (16.67)	30 (15)	2.57(1.16-5.71)	**0.03**
AG CT	65 (36.11)	80 (40)	2.09(1.04-4.2)	**0.04**
AG TT	11 (6.11)	06 (3)	4.71(1.46-15.20)	**0.009**
GG CC	18 (10)	06 (3)	7.71(2.54-23.44)	**0.0001**
GG CT	14 (7.78)	02 (1)	18(3.61-89.61)	<**0.0001**
GG TT	03 (1.67)	0	-	**0.02**

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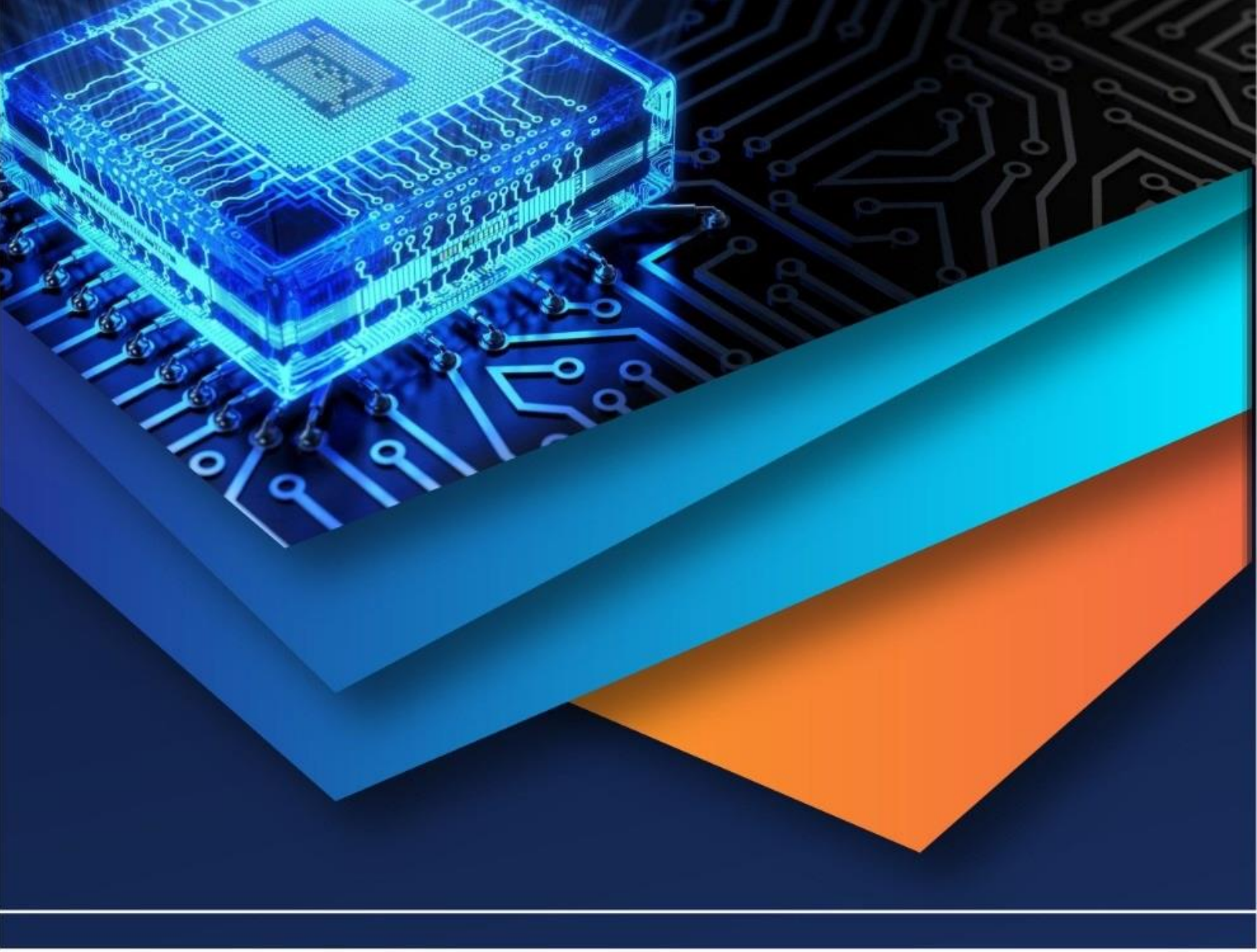

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