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TP53 MUTATIONS IN BREAST CANCER TUMORS OF PATIENTS FROM RIO DE JANEIRO, BRAZIL: ASSOCIATION WITH RISK FACTORS AND TUMOR CHARACTERISTICS

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Somatic mutations in the *TP53* gene are the most frequently observed genetic alterations in human malignancies, including breast cancer, which is one of the leading causes of death among women in Brazil. In our study, we determined the frequency and the pattern of *TP53* mutations in malignant breast tumors from 120 patients living in Rio de Janeiro, Brazil. *TP53* mutations were found in 20% of the tumors, which contained a diversity of mutation types: missense (62.5%), nonsense (8.3%), silent (4.2%), intronic (12.5%), insertion (4.2%) and deletion (8.3%). Of a total of 15 missense mutations, 4 were observed at Arg248 and 2 at Cys242, which are directly involved in DNA binding and in zinc binding, respectively. A subgroup of 51 patients was analyzed with respect to the relation between the presence of *TP53* mutations and classical risk factors and with tumor and patient characteristics. For this analysis, we used logistic regression and, in order to obtain more precise confidence intervals, they were recalculated using a bootstrap resampling technique. Our results demonstrate that these mutations are not statistically associated with the risk factors or the patients' characteristics. However, the presence of *TP53* mutations is strongly associated with the aggressiveness of the tumors, measured by Elston classification (OR = 11.97 and 95% CI of 2.24–307.05). This finding is in agreement with previous studies, which report the presence of *TP53* mutations in tumors with poor prognosis. This correlation between tumor aggressiveness and *TP53* mutations could be a crucial variable for the treatment and prognosis of breast cancer.

Key words: *TP53 gene; breast cancer; risk factors; clinicopathologic features; Rio de Janeiro, Brazil; Elston grade*

Breast cancer is one of the leading causes of death among women in Brazil. The Brazilian expected mortality rate for 2001 is 9.9 women per 100,000, whereas the highest incidence was estimated to be in the State of Rio de Janeiro: 82 women per 100,000.[1] The etiology of breast cancer is rather complex and, although 10–15% of the patients have a family history of the disease, only a small proportion can be explained by mutations in genes such as *BRCA1* and *BRCA2*.

The different geographic distribution of cases and evidence from migration studies suggest that some environmental and/or lifestyle factors may be related to the development of breast cancer.[2] Reproductive history, family history of breast cancer, cigarette smoking and alcohol consumption are generally cited as risk factors of breast cancer. However, how these factors contribute to trigger the molecular mechanisms of tumor initiation and progression is not completely understood.[3]

Mutations in the tumor suppressor gene, *TP53*, are the most common genetic alterations seen in human cancer.[4] This gene encodes a 393 amino acid nuclear phosphoprotein that acts as a transcription factor and is implicated in nearly all pathways involved in cell proliferation control: modulation of cell-cycle progression, apoptosis, DNA repair, cell differentiation and senescence.[5] The type and location of mutations in *TP53* are different in distinct human cancers. This variability and the mutation spectrum may represent etiologic factors acting in the malignant transformation process.[4]

In breast cancer, the occurrence of *TP53* mutations is described to range from 12–60% of all tumors.[4,6] Furthermore, the presence of *TP53* mutations corresponds to a more aggressive tumor phenotype indicating that the analysis of the mutation pattern may be an interesting tool to study possible correlations with etiologic agents as well as prognostic factors in breast tumors.[7]

In our study, we determine the type and location of *TP53* mutations among Brazilian breast cancer patients who are residents of Rio de Janeiro City. *TP53* mutations were associated with classic risk factors for breast cancer and with the tumor and patients' characteristics. To our knowledge, this is the first detailed study of *TP53* mutation spectrum in breast cancer in Brazil.

MATERIAL AND METHODS

Study subjects and interviews

All of the studied patients ($n = 120$) were admitted for invasive breast cancer surgery at the National Institute of Cancer, in Rio de Janeiro, from 1995–1997. Fifty-one patients from the group of 120 were interviewed using a structured questionnaire in order to perform a strict analysis of the possible relationship between *TP53* mutations and epidemiologic factors or tumor characteristics. Women from this group were eligible for the study only if they lived in the metropolitan area of Rio de Janeiro and were younger than 75 years old. Interviewers, specially trained for the study, elicited detailed information about the patient's residential area, socioeconomic status, educational level, and about the selected risk factors. The variables included in our study were age, parity status, lactation, family history of breast cancer and other cancers (only for first-degree relatives), tobacco smoking (ever and never) and alcohol consumption. Cancer diagnoses were confirmed histopathologically and 94% of the tumors were diagnosed as invasive ductal carcinoma. Tumor characteristics, grade, size and nodal

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status were obtained from hospital records. The tumors were classified using the Elston grade system.[8] All patients had primary tumors and were not treated with chemotherapy or radiotherapy before the surgery. The Ethics Committee of the National Institute of Cancer approved the study proposal and all ethic proceedings.

DNA isolation and PCR-SSCP

Breast cancer tissues were collected after surgery, frozen in liquid nitrogen and stored at −70°C. Genomic DNA from the tumors was prepared with proteinase K digestion and phenol/chloroform extraction according to standard procedures.[9] Exons 5–8 of the *TP53* gene were amplified using intronic primer pairs, as described elsewhere.[10] Briefly, PCR was carried out with 500 ng of DNA, 13 pmoles of each primer, 0.2 mM dNTP, 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 1.5 mM $MgCl_2$ and 1.5 U of *Taq* DNA polymerase in a final volume of 50 μl. PCR cycling conditions were carried out with an initial denaturation step for 5 min at 94°C, followed by 30 cycles consisting of 3 steps: 94°C for 30 sec, 61°C for 1 min and 72°C for 1 min. A final extension step was performed at 72°C for 10 min.

The SSCP analysis was originally described by Orita *et al.*:[11] PCR products (5 μl) were diluted 2-fold with a formamide denaturing buffer (95% formamide, 100 mM EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol and 50% glycerol), heated at 95°C for 5 min and loaded on a nondenaturing 8% polyacrylamide gel. Gels were run in 1× Tris-borate-EDTA buffer for 18 hr at room temperature. Gels were silver stained as described elsewhere.[12]

Direct sequencing

Tumor samples showing an abnormal band shift in 3 independent PCR-SSCP experiments were considered to contain a mutation. Tumor DNA that contained mutations was used in a new PCR reaction and the amplified product corresponding to the mutated region of *TP53* was run on a 1.5% agarose gel. The band was excised, purified with the Sephaglas™ BandPrep Kit (Amersham Pharmacia Biotech, Piscataway, New Jersey) and sequenced. Direct sequencing was performed with dideoxy nucleotide chain terminators in cycle sequencing reactions with *Taq* DNA polymerase as described by Lee,[13] using 1 μCi of α-[P^{33}]d-ATP (3000Ci $mmol^{-1}$) (Amersham Pharmacia Biotech).

Statistical analysis

Kruskal-Wallis test was used to compare age, educational level and menopausal status of patients with and without *TP53* mutations. The association between *TP53* mutations and the studied variables was evaluated through logistic regression procedures. To obtain more precise confidence intervals, a large number of new data sets were created by the bootstrap method, which is based on randomly resampling from a given experimental sample, in order to simulate the effect of using multiple samples from the same population.[14,15] Age-adjusted odds ratios (OR) and 95% confidence intervals (CI) of 1,000 new data sets resampled from the initial data were calculated to evaluate the association between *TP53* mutations and the studied variables. STATA software pV 6.0 was used for all analysis.

RESULTS

Description of the TP53 *mutations*

PCR-SSCP analysis of exons 5–8 of the *TP53* gene showed 24 mutated samples among the 120 breast cancer cases (20%) (Table I). Eleven alterations in exon 5, 4 in exon 6, 10 in exon 7 and 1 in exon 8 were found. Two tumors contained alterations in 2 exons: one tumor had alterations in exons 7 and 8 and the other in exons 5 and 7. All alterations observed by the PCR-SSCP analysis were confirmed by DNA sequencing. Figure 1 shows a representative SSCP analysis and its corresponding sequencing gel. Among the mutations, 21 (80.8%) were point mutations, of which 15 (57.7%) were missense, 2 (7.7%) were nonsense, 1 (3.8%) was a silent mutation and 3 (11.6%) were in intron sequences. One intronic mutation was localized between exons 6 and 7, and the other 2 between exons 7 and 8. The other 4 alterations were 2 (7.7%) deletions, 1 (3.8%) insertion and 1 polymorphism. This polymor-

TABLE I – SITE AND SEQUENCE CHANGE OF *TP53* MUTATIONS IN BREAST CANCER TUMORS

Patient number[1]	Exon/intron	Codon	Nucleotide change[2]	Change	
				Amino acid	Site[3]
Missense					
05	5	175	CGC→CAC	Arg→His	L2/III
10	5	151	CCC→CAC	Pro→His	
15	5	144	CAG→CAC	Gln→His	β strand-3
25	7	255	ATC→ACC	Ile→Thr	β strand-9/IV
26	7	230	ACC→TCC	Thr→Ser	β strand-8
27	7	248	CGG→TGG	Arg→Trp	L3/IV/DNA
29	7	248	CGG→CCG	Arg→Pro	L3/IV/DNA
39	7	248	CGG→TGG	Arg→Trp	L3/IV/DNA
41	7	242	TGC→AGC	Cis→Ser	L3/IV/Zn
60	7	242	TGC→AGC	Cis→Ser	L3/IV/Zn
62	6	201	TTG→CCG	Leu→Pro	
68	5	146	TGG→CGG	Trp→Arg	β strand-3
107	5	131	AAC→AGC	Asn→Ser	β hairpin/II
107	7	248	CGG→TGG	Arg→Trp	L3/DNA
118	5	151	CCC→CAC	Pro→His	
Nonsense/deletion/insertion					
13	8	306	CGA→TGA	Arg→STOP	
136	5	141	TGC→TGA	Cis→STOP	β hairpin/II
07	5	130–142	37 bp del.	Frameshift	β hairpin/II
125	5	173–178	16 bp del.	Frameshift	L2/III
134	5	137–138	1 bp ins.	Frameshift	β hairpin/II
Intronic/silent/polymorphism					
13	5	Intron	ctg(t→c)gc		
44	7	Intron	gag(g→a)tc		
64	7	Intron	gcc(a→t)cc		
19	6	175	CGC→CAG	His→His	β strand-5
111	6	213	CGA→CGG	Arg→Arg	

[1]Patients 107 and 13 have 2 mutations: the former in codon 131 and 248 and the latter in codon 306 and intron 5.–[2]Nucleotide change: del., deletion; ins., insertion.–[3]Site: L2, loop 2; L3, loop 3; Zn, zinc-binding domain; DNA, direct contact with DNA; II, III and IV, conserved regions.

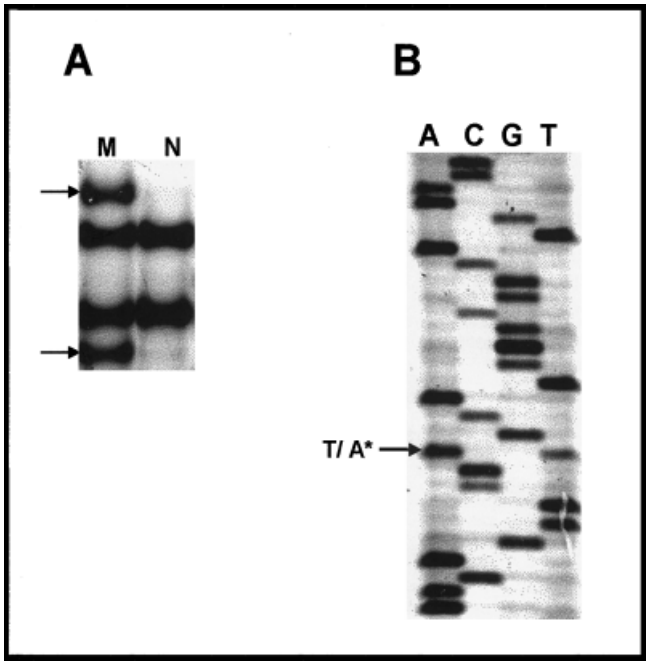

FIGURE 1 – Representative SSCP and sequencing analysis of exon 7 of *TP53* from patient 60. *(a)* SSCP: N is normal DNA that has wild-type *TP53* and M is tumor DNA that shows a mutation indicated by the band shift (arrows). *(b)* Direct sequencing: The arrow indicates a base substitution (T→A*) in exon 7 of *TP53*.

phism was detected in exon 6, at codon 213, and was not considered in the mutation analysis.

Among the point mutations, 12 (57.1%) were transitions, mostly C→T (41.7%). Eighty percent of C→T transitions occurred at a CpG site. Nine (42.9%) of the point mutations were transversions. Most mutations occurred within conserved domains II, III and IV of the protein, especially in the DNA and zinc-binding domains, at codons 248 and 242, respectively, and in the S2-S2′β hairpin domain, codons 130–141 (Table I).[16]

Association of TP53 *mutations with the tumors and the patients' characteristics*

The frequency of *TP53* mutations was quite similar between the larger group ($n = 120$) and the group of patients that was interviewed ($n = 51$); 20% in the first group and 19.6% in the second group. The difference between the 2 groups was not significant and all statistical analyses were done with the later group.

The age, educational level and menopausal status of the breast cancer patients are presented in Table II. In relation to age at the time of surgery, we observed that 4 (40%) of the 14 women younger than 50 and 6 (24.4%) of the 37 women older than 50 had *TP53* mutations. The difference in the mean age of these patients with and without *TP53* mutations (55.7 and 57.3 years old, respectively) was not statistically significant ($p = 0.51$, Kruskal-Wallis). The 2 groups did not show any differences with respect to the educational level or menopausal status (Table II), demonstrating that the 2 subgroups—mutated or not mutated—are homogenous in relation to the patients' characteristics.

In relation to the tumor characteristics, 3 relevant factors were analyzed: Elston grade, tumor size and nodal status. Interestingly, among these factors, Elston grade was significantly associated with the presence of mutations. This association is shown in Table III (OR = 11.97; 95% CI 2.24–307.05).

TP53 *mutations' association with risk factors*

The *TP53* mutation spectrum was analyzed in relation to the patients' risk factors: family history of breast cancer (first-degree relatives) or other cancers, parity, lactation, tobacco smoking and alcohol consumption. In relation to family history of breast cancer, of 50 women, 2 (4%) had breast cancer in first-degree relatives. The association between family history of cancer and altered *TP53* was not statistically significant (Table IV), but it showed a trend to this risk factor (OR = 4.8, 95% CI 0.92–25.13). We could not find any associations between *TP53* mutations and the other risk factors analyzed (Table IV).

TABLE II – DESCRIPTIVE CHARACTERISTICS OF THE 51 BREAST CANCER PATIENTS

Characteristics	*TP53* mutation		Total
	Negative	Positive	
Age at diagnosis			
30–39	1	1	2
40–49	9	3	12
50–59	14	1	15
60–69	10	4	14
70–75	7	1	8
	(p = 0.32)		
Educational level			
Illiterate	4	3	7
Elementary school	17	3	20
Junior high school	7	1	8
Senior high school	7	2	9
College	6	1	7
	(p = 0.63)		
Menopausal status			
Postmenopause	33	7	40
Premenopause	8	3	11
	(p = 0.67)		

TABLE III – ASSOCIATION BETWEEN *TP53* MUTATIONS AMONG BREAST CANCER PATIENTS AND TUMOR CHARACTERISTICS

Tumor	Characteristics	(no. of patients)	Age-adjusted OR	95% CI
	Elston grade[1] (48)			
TP53 mutation	I and II	III		
Negative	33	5	1.00	
Positive	4	6	11.97	(2.24–307.05)
	Tumor size (mm)[2] (49)			
TP53 mutation	<20	≥20		
Negative	7	1	1.00	
Positive	34	7	1.20	(0.12–3.01)
	Nodal status[1] (48)			
TP53 mutation	Negative	Positive		
Negative	17	2	1.00	
Positive	23	6	2.20	(0.46–10.58)

[1]Three cases were missing.–[2]Two cases were missing.–OR, odds ratio; CI, confidence interval.

DISCUSSION

The study of *TP53* mutations in human cancers is undoubtedly important. However, with respect to breast cancer, a relationship between the presence of *TP53* mutations and prognostic factors is not clear. In our study, we analyzed the *TP53* mutational status of breast cancer in patients from Rio de Janeiro, Brazil, and its relationship to well-known breast cancer risk factors and with relevant tumor characteristics. The *TP53* mutation spectrum (Table I) and frequency (20%) of these tumors are in accordance to what was observed in other studies of sporadic breast cancers.[4,6,7,17]

This is the first time the *TP53* mutational spectrum of Brazilian breast cancer patients has been published. As described by Hainaut[4] and others,[7,18] mutations in *TP53* may indicate the presence and action of carcinogens and could help us to understand the breast cancer etiology in patients living in Rio de Janeiro. The

TABLE IV – ODDS RATIOS OF *TP53* MUTATION AMONG BREAST CANCER PATIENTS ASSOCIATED WITH SELECTED RISK FACTORS

Risk factors (no. of patients)	*TP53* mutation		Age-adjusted OR	95% CI
	Negative	Positive		
Parity status (51)				
Nulliparous	7	2	1.00	
Parous	34	8	0.85	(0.16–4.64)
Breastfeeding (51)				
No	9	2	1.00	
Yes	32	8	1.23	(0.22–6.79)
Family history of breast cancer[1] (50)				
No	40	8	1.00	
Yes	1	1	4.80	(0.92–25.13)
Family history of all types of cancer[1] (50)				
No	30	6	1.00	
Yes	11	3	1.44	(0.26–2.08)
Tobacco smoking (51)				
No	23	8	1.00	
Yes	18	2	0.30	(0.07–1.32)
Alcohol consumption (51)				
No	24	7	1.00	
Yes	17	3	0.63	(0.12–1.20)

[1]Family history of breast and all types of cancer information for 2 patients are missing (first-degree relatives were considered).–OR, odds ratio; CI, confidence interval.

occurrence of breast cancer in Southeastern Brazil, where Rio de Janeiro City is located, is similar to that presented by many other cities in developed countries.[1] This is probably related to the similarity in life habits.

The most common type of *TP53* mutations found in breast cancers is point mutations, mostly C→T transitions at CpG sites.[19] This cytosine is frequently methylated in mammalian cells, leading to the formation of a U:G mismatch by spontaneous deamination. Our results showed the same pattern that is, in fact, common to a great number of tumors.[18] Six of 15 patients with missense mutations had alterations in amino acids directly involved in DNA (Arg248) and zinc binding (Cys242), located in loop 3 of the protein central domain.[20] Finally, in an overall analysis, we did not find relevant differences between the mutation pattern described in our study and other studies found in the IARC *TP53* mutation database (http://www.iarc.fr/p53).

The development of breast cancer involves multiple possible acting factors in its etiology,[2] and any clue obtained from experimental data may contribute to the elucidation of the process. This study is the first to perform a detailed analysis of the mutation spectrum of *TP53* in breast tumors in Brazil. In addition, we decided to do a more accurate analysis of *TP53* mutations in relation to selected risk factors and some relevant tumor characteristics in a group of 51 patients. This same group of patients took part in a recently published case-control study,[21] where data were collected by specially trained interviewers. In this set of patients, the *TP53* mutation frequency (19.6%) was maintained when compared to the larger group (20%). This is in agreement with other studies that have observed frequencies of 12–60%.[4,6] Our results showed that the presence of *TP53* mutations in breast tumors from patients of Rio de Janeiro is not statistically related to the studied risk factors. We found that the OR tended to history in relation to the family risk of breast cancer, although without statistical significance. This may be explained by the interference of other altered genes that confer susceptibility, such as *BRCA1*, which were not analyzed in our study. In this regard, *TP53* mutations are more frequent in tumors from patients presenting family history of breast cancer associated with *BRCA1/2* mutations as described by Greenblatt *et al.*[22] Another possibility is that familial tendency may be associated with genes of carcinogen metabolizing enzymes, for which there are many existing polymorphisms, which could participate in the mechanisms that contribute to the induction of *TP53* mutations. This issue is currently under investigation in our laboratory.

An important aspect that comes out of our study of the *TP53* mutation pattern is related to the clinical perspectives. Several studies have shown significant associations between *TP53* mutations and poor prognosis of the breast tumor.[20,23,24,25] Therefore, we investigated whether the presence of *TP53* mutations is associated with breast tumor characteristics: Elston grade, tumor size and nodal status. The Elston grade is currently used by pathologists as a classification of the malignant grade of breast tumors that indicates the aggressiveness of the tumor and worst survival for the patients at grade III. This method of classification is derived from the assessment of 3 morphologic features: tubule formation, nuclear pleomorphism and mitotic counts.[8] Our results demonstrated a strong association with Elston-grade classification but not with tumor size and axillary lymph nodes involvement. Tumors classified as grade III were related to a greater number of mutated tumors (6 in 11), which correspond to 54% of the cases. These analyses were performed with the larger group and showed similar results: 50% of the mutated tumors belonged to the most aggressive type, Elston grade III (data not shown). Interestingly, 4 of 6 tumors with missense mutations in the zinc-binding domain or in the codon 242 that directly contacts DNA were of grade III. These results agree with other studies, which observed a correlation between these types of mutations and the aggressiveness of the breast tumor.[24,25] In another study, Berns *et al.*[24] observed that these mutations in amino acids located in loop 3 of the protein's central domain were related to an even poorer response to the antiestrogen tamoxifen, one of the major compounds used for the endocrine treatment of breast cancer.

Recent findings show that missense mutations in *TP53* can inactivate its function as a sequence-specific transactivator and lead to the expression of a full-length mutant protein with a single amino acid substitution.[26] Mutant p53 can act as an oncogene by binding to the nuclear matrix and to DNA at the matrix attachment regions, causing alterations in the nuclear structure and eliciting cell proliferation and enhanced tumorigenicity.[26] The degradation of the mutant protein is markedly reduced within tumor cells, which accumulate high amounts of mutated p53. A possible explanation for the correlation between the presence of *TP53* mutations and tumor aggressiveness is that the mutations that lead to an oncogenic form of p53 can be selected during tumor development, conferring an advantage to tumor cell growth and/or aggressiveness.

In a meta-analysis, Pharoah *et al.*[27] showed that for primary breast cancers, somatic *TP53* mutations are strong independent

markers for survival. We are now collecting data related to these patients' survival. Alsner *et al.*[20] showed that tumor characteristics and clinical outcome are related to the presence, type and location of *TP53* mutations. Our data reinforces that mutations in *TP53* are important parameters to be taken into account for clinical measures.

In conclusion, our work identifies for the first time the *TP53* mutation pattern in breast tumors from patients of Rio de Janeiro City, Brazil, and demonstrates that these mutations are not statistically associated with classical risk factors but are strongly associated with the aggressiveness of the tumors. The type of *TP53* alterations and the affected region of the protein could be of considerable prognostic value and might be useful to direct clinicians on how to design a treatment protocol suited to each patient.

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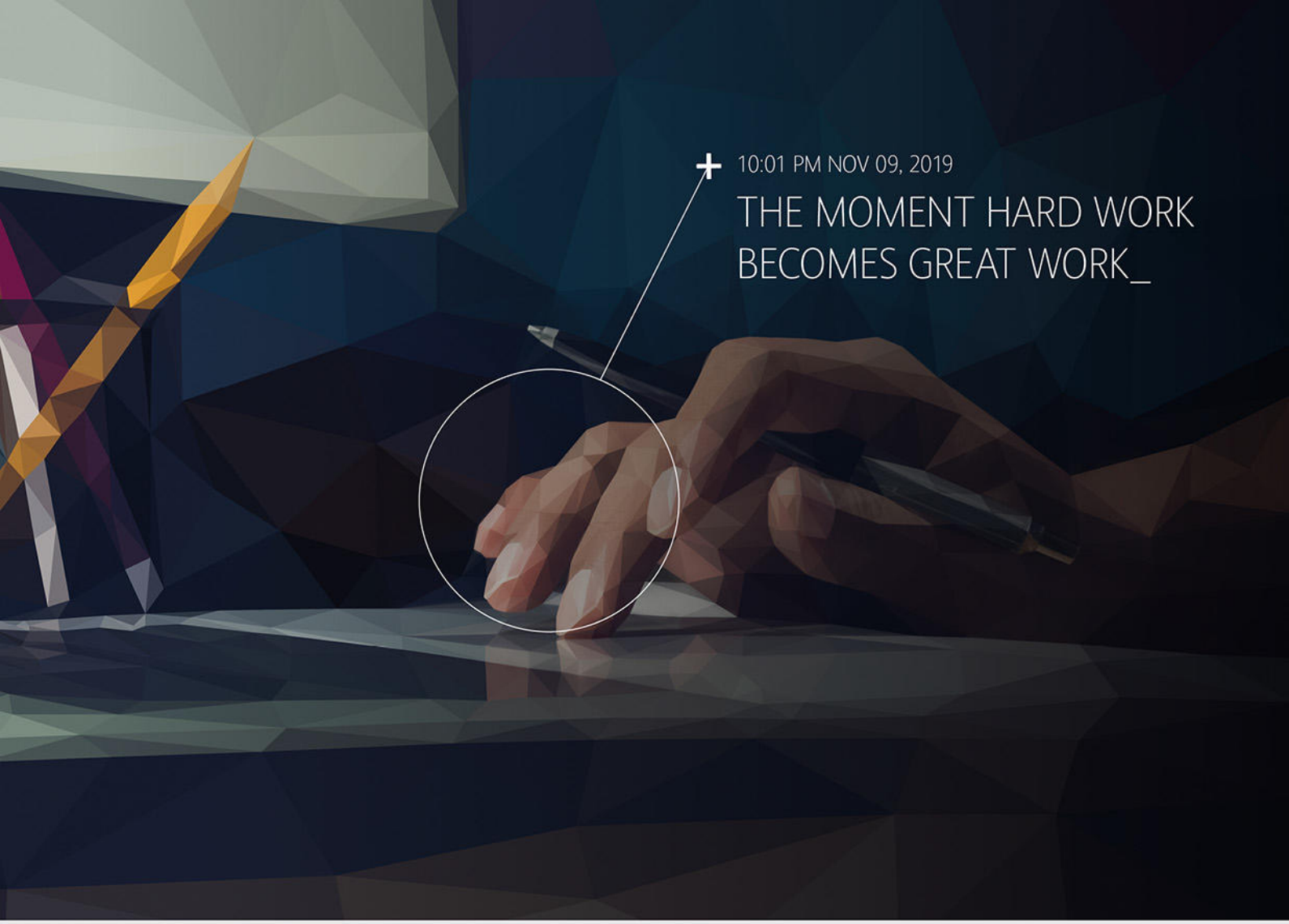

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