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Title

Evaluating RB1 and p53 as Diagnostic Markers in Treatment-related Neuroendocrine Prostate Cancer through Immunohistochemistry and a Genomic Analysis of *RB1* and *TP53*

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The data supporting the findings of this study are available within the article and its supplementary materials.

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Conflict of Interest Disclosure

The authors declare no conflicts of interest.

Ethics Approval Statement

This study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board (IRB) of Kobe University Hospital (approval number: B220013).

Patient Consent Statement

Informed consent for this study was obtained through an opt-out methodology.

Permission to Reproduce Material from Other Sources

No material from other sources was reproduced in this study.

List of abbreviations

PC = prostate cancer

ADT = androgen-deprivation therapy

CRPC = castration-resistant prostate cancer

NEPC = neuroendocrine prostate cancer

t-NEPC = treatment-related neuroendocrine prostate cancer

SCC = small-cell carcinoma

PSA = prostate-specific antigen

NCCN = National Comprehensive Cancer Network

NE = neuroendocrine

NGS= next-generation sequencing

IHC = immunohistochemistry

SNP = synaptophysin

CgA = chromogranin A

FFPE = paraffin-embedded

ARSI=androgen receptor signaling inhibitor

NSE = neuron-specific enolase

Pro-GRP = pro-gastrin-releasing peptide

CEA = carcinoembryonic antigen

Abstract

Background

The diagnosis of treatment-related neuroendocrine prostate cancer (t-NEPC) often involves a pathological assessment and immunohistochemistry (IHC) for neuroendocrine markers. Genomic alterations in *RB1* and *TP53* are frequently observed in NEPC and are believed to play a crucial role in the transformation of adenocarcinoma to NEPC. In this study, we examined the clinicopathologic, immunohistochemical, and genetic features of patients with t-NEPC to better understand their prognosis and diagnostic utility.

Methods

This retrospective study reviewed the records of patients diagnosed with t-NEPC at Kobe University Hospital between October 2018 and December 2022. Clinical data, including the age, serum neuroendocrine marker levels, and treatment history, were collected. IHC was performed for conventional neuroendocrine markers (synaptophysin, chromogranin A, and CD56) and RB1 and p53 expression. Next-generation sequencing (NGS) was conducted using FoundationOne® CDx to identify mutations in RB1 and *TP53*.

Results

This study included 20 patients with t-NEPC. The median time from ADT initiation to the development was 42.8 months. IHC revealed RB1 loss in 75% of cases and p53 abnormalities in 75% of cases. NGS identified *RB1* mutations in 55% and *TP53* mutations in 75% of cases. The concordance between NGS and IHC results was high, with 70% (14/20) agreement for RB1/*RB1* and 80% (16/20) for p53/*TP53*. The immunostaining and genomic analysis of RB1/*RB1* and p53/*TP53* showed abnormal findings for the 4 negative cases for conventional neuroendocrine markers.

Conclusions

This study indicated high concordance between IHC and NGS findings for RB1/*RB1* and p53/*TP53* in t-NEPC. We provide a comprehensive benchmark of NGS performance compared with IHC, and these findings may help increase the diagnostic sensitivity of t-NEPC.

Keywords: NEPC, RB1, p53, immunohistochemistry, Diagnostic marker

Introduction

Prostate cancer (PC) is the most common cancer in men and the leading cause of cancer-related death in developed countries[1]. The standard treatment for metastatic PC is androgen deprivation therapy (ADT); however, tumors often acquire resistance, and castration-resistant prostate cancer (CRPC) eventually develops. Neuroendocrine prostate cancer (NEPC) has an aggressive phenotype, is associated with a poor prognosis, and can arise *de novo* or as treatment-related NEPC (t-NEPC) as a resistance mechanism in patients previously treated with ADT. Although the incidence of pure *de novo* small-cell carcinoma (SCC) is low (<2%)[2,3], t-NEPC is more common. A recent study of autopsy samples showed that up to 25% of patients who died of CRPC had signs of t-NEPC[4,5]. Unresponsiveness to hormone therapy, presence of lytic bone lesions, rapid disease progression, visceral metastases, marked prostatic enlargement, and disproportionately low prostate-specific antigen (PSA) levels in the setting of metastatic disease are key clinical features of NEPC. Because of these clinical implications, the National Comprehensive Cancer Network (NCCN) has recently updated its guidelines to include consideration of a metastatic biopsy in patients with CRPC to evaluate t-NEPC transformation[6].

The most commonly used findings for diagnosing t-NEPC are undeniably pathological findings. For decades, immunohistochemistry (IHC) has been used to reveal foci of neuroendocrine (NE) cells in conventional prostate adenocarcinomas, a pathological variant known as adenocarcinomas with NE differentiation. NEPC is generally diagnosed comprehensively, considering the morphology of the tissue and the results of immunostaining for NE markers (synaptophysin (SNP), chromogranin A (CgA), and CD56). However, accurately distinguishing small-cell carcinoma from high-grade acinar prostate carcinoma based on morphology alone can be difficult, and the interpretation of NE markers is often challenging due to their staining patterns.

Numerous studies of small cell carcinomas arising in other organs have suggested that

the loss of *RB1* and *TP53* genes is an extremely common event in this tumor type in humans, and these changes are sufficient to generate small cell carcinomas in the lungs of mice[7–9]. In prostate cancer, multiple preclinical studies have supported combined *RB1* and *TP53* deficiency as key facilitators of the NE-like phenotype[10–13]. Alterations, such as *RB1* and *TP53* deficiency, which are enriched in NEPC tumors, are often acquired during the course of therapy resistance. With the widespread use of next-generation sequencing (NGS), genomic analyses have become increasingly common in cases of suspected NEPC, providing more opportunities to identify mutations in *RB1* and *TP53*. However, whether or not an IHC evaluation is necessary if a genomic analysis is performed and whether there is any benefit from evaluating aberrant expression of RB1 and p53 by IHC remain unclear.

IHC methods for evaluating RB1 and p53 expression have been well established in pathology practice. However, benchmarking of NGS and IHC in evaluating RB1 and TP53 in NEPC has not been previously performed.

To address this knowledge gap, the present study analyzed the differences between immunohistochemical and genomic analysis findings for RB1/*RB1* and p53/*TP53* and combined them with the results of conventional NE markers to explore their clinical significance.

Materials and Methods

Patient cohort

This retrospective study evaluated the records of consecutive patients diagnosed with t-NEPC at the Kobe University Hospital between October 2018 and December 2022. Patients with clinically suspected NEPC based on a prior history were diagnosed with t-NEPC after undergoing a biopsy of the sites of disease progression, such as the prostate region and metastases. Clinical and demographic data, including age, sex, serum NE tumor marker levels, pathological prostate grade, clinical stage, sites of metastasis, and treatment progress, were collected from medical records. Validation data for RB1/*RB1* and p53/*TP53* in prostate adenocarcinoma and CRPC were obtained from cBioPortal for Cancer Genomics and The Cancer Genome Atlas Program (TCGA). Clinical stage was determined based on the 2017 TNM Classification of Malignant Tumors, 8th Edition[14].

SCC-type NEPC was defined according to the morphologic criteria established for small-cell lung tumors, namely, small- to medium-sized cells with a minimal amount of cytoplasm and hyperchromatic overlapping nuclei with no or few nucleoli. Mixed NEPCs and *de novo* NEPCs with adenocarcinoma components were excluded from this study. Standardized regimens of EP (etoposide 100 mg/m^2 on days 1–3 plus cisplatin 80 mg/m^2 or carboplatin 5 [mg/mL·min] × [GFR mL/min + 25] on day 1 every 3 weeks) were administered as the first-line treatment in all cases.

IHC and validation

IHC for conventional NE markers (including SNP, CgA, CD56, and INSMI) diagnostic of NEPC was evaluated by a pathologist (N.J.) using the H-score (0–300), which was defined as the multiplication of intensity (0, 1=weak, 2=moderate, 3=strong) and proportion (0% to 100%) of the expressed tumor cells according to a previously reported study[15,16]. IHC staining of the

tissue sections was used as a diagnostic reference and was performed using primary antibodies against SNP (MRQ-40; Santa Cruz Biotechnology, Dallas, TX, USA), CgA (DAK-A3; Dako, Hamburg, Germany), CD56 (MRQ-42; Roche, Basel, Switzerland), and INSMI (A-8; S Santa Cruz Biotechnology).

RB1 and p53 expressions were examined using whole slides in all cases. RB1 immunostaining was performed using BOND-MAX (Leica Biosystems, Nussloch, Germany) with pre-diluted antibodies against clone G3-245 (RUO) (BD Biosciences, Franklin, NJ, USA). The expression of RB1 was divided into 2 categories: loss, absence of ≥90% RB1, and wild-type expression, which does not fit the definition of loss. p53 immunostaining was performed using a Ventana BenchMark GX (Roche) with prediluted antibodies against clone DO-7 (Dako Agilent, Santa Clara, CA, USA). The expression of p53 was divided into 3 categories: overexpression, ≥80% indicated strong and diffuse nuclear positivity (regardless of the presence of cytoplasmic expression), complete absence, absence of ≥90% p53, and wild-type expression.

NGS

NGS was performed on the tissue collected from the biopsy. Comprehensive genomic analyses were performed using FoundationOne® CDx (F1CDx; Foundation Medicine, Inc., Cambridge, MA, USA). Formalin-fixed, paraffin-embedded (FFPE) samples retrieved from tumor specimens were collected, and 10 unstained sections and 1 hematoxylin and eosin-stained (H&E) section were delivered to Foundation Medicine, Inc. FoundationOne® CDx applies NGS across 324 genes known to be drivers of solid tumors with high accuracy, by sequencing the coding regions of 309 cancer-related genes and introns from 36 genes. Sequencing was performed using an Illumina HiSeq® 4000 (Illumina, San Diego, CA, USA) to identify base substitutions, insertions and deletions (indels), copy number alterations (CNA), and

rearrangements. FoundationOne® was able to cover the entire exonic region of *RB1* and *TP53* for mutation determination.

PFS was defined as the time between the date of treatment initiation and disease progression or death from any cause. Patients who were alive at the date of last follow up were censored. Kaplan–Meier curves were constructed to analyze PFS and all statistical analyses were conducted using the EZR software program (Saitama Medical Center, Jichi Medical University, Japan). Statistical significance was set at $P<0.05$.

Ethical statement

The Institutional Ethical Committee approved the study protocol (approval number: B220013). Informed consent was obtained using an opt-out approach.

Results

Among all patients, 23 cases of t-NEPC were present, but 3 mixed NEPC were excluded. The clinicopathological characteristics of the 20 included patients with primary adeno-PC before conversion to t-NEPC are shown in Table 1. At the time of the first prostate biopsy, the median PSA level was 58.8 (range 4.2–394) ng/ml; 18 (90.0%) patients had Gleason score ≥8 pure adenocarcinoma, and 13 (65.0%) patients had metastatic disease. An androgen receptor signaling inhibitor (ARSI) was introduced in 45.0% (9/20) of the patients in the pretreatment group.

The clinicopathological characteristics of patients after t-NEPC development are shown in Table 2. The median time from ADT initiation to t-NEPC development was 42.8 months. The median neuron-specific enolase (NSE) was 44.7 (range 6–677) ng/ml, and the median pro-gastrin-releasing peptide level (GRP) was 60.5 (range 29–50000) pg/ml. NSE, pro-GRP, and carcinoembryonic antigen (CEA) levels were elevated in 60.0% (12/20), 40.0% (8/12), and 45.0% (9/12) of patients, respectively. SNP was positive in most cases, while CgA was low, with H-scores below 100 in all but 4 cases (Table 2).

Genomic analysis revealed *RB1* mutations in 55.0% (11/20) of cases. Using IHC, loss of RB1 was observed in 75.0% (15/20) of the cases, whereas 25% (5/20) showed retained RB1 expression (Table 3). Based on NGS and IHC data, the following groups were identified: RB1 loss/*RB1*-mutated, 50% (10/20); RB1 wild/*RB1*-mutated, 5% (1/20); RB1 loss/*RB1*-normal, 20% (4/20); and RB1 wild/*RB1*-normal, 25% (5/20) (Figure 1). *TP53* mutations were identified in 75.0% (15/20) of the cases. IHC revealed that 40.0% (8/20) of the patients had p53 loss, and 35.0% (7/20) had p53 overexpression. When limiting the analysis to genomic abnormalities that can significantly affect protein function, such as deletions and frameshifts, 91.2% (11/12 cases) of the genomic analysis and immunostaining assessments were consistent in RB1/*RB1* and p53/*TP53*.

Table 4 shows the NGS and IHC results for each patient. Case 8 was negative for NE markers (SYN, CgA) and had no *RB1* mutation on NGS but had RB1 loss and p53 overexpression on IHC. Cases 16, 18, and 20 also had low H-scores for all NE markers and were also hetero in IHC for RB1. However, IHC revealed that p53 was lost or overexpressed, and genomic mutations were also observed. IHC revealed that three cases of RB1 loss, two cases of p53 loss, and five cases of p53 overexpression showed abnormal expression before NEPC transformation. The objective response rate to first-line EP was 55% (1 complete response and 10 partial responses).

Figure 2 shows the results of PFS stratified by NGS and IHC findings. There was no significant difference between the patients with and without mutations in both *RB1* and *TP53*. PFS was also examined in patients with and without abnormalities in both RB1 and p53 by IHC, and no significant difference was found in this group.

Discussion

This study analyzed genomic mutations using exon sequencing and IHC for RB1/*RB1* and p53/*TP53* proteins in 20 patients diagnosed with t-NEPC. We provide a comprehensive benchmark for NGS performance compared to IHC. This study had two key findings. First, there was high concordance between RB1/*RB1* and p53/*TP53* genomic mutations and IHC findings. Second, assessing RB1 and p53 levels using IHC may help diagnose t-NEPC.

The increased use of ARSI may lead to more frequent encounters with t-NEPC[17]. Given its poor prognosis, an early diagnosis and appropriate platinum-based treatments are crucial. Although a morphological pathological evaluation is fundamental for diagnosing NEPC, NE markers are typically used as adjuncts. NE markers, such as SYN, CgA, and CD56, do not comprise an optimal marker panel for small-cell differentiation in prostate cancer, as they can be seen in up to 100% of acinar carcinomas in some series[18], indicating that current immunohistochemical markers for NE differentiation may be non-specific[19]. Therefore, recent reports have focused on RB1 and p53 as markers of tumors with NE features. Although these two genetic abnormalities are not necessary or sufficient conditions for t-NEPC[20], the combined loss of RB1 and p53 is suggested to promote lineage plasticity. In particular, RB1 loss is associated with a poor prognosis in CRPC[21]. The singular or combined loss of these tumor suppressors may predict transformation to NEPC and help identify patients at a high risk of developing this condition[22]. Beltran et al. reported that the concurrent loss of *RB1* and *TP53* was present in 53.3% of tumors in NEPC patients and 13.7% of CRPC adenocarcinoma samples[23]. Furthermore, Tan et al. found that Rb loss occurs almost universally at the protein level in SCC, suggesting that loss of this protein may serve as a valuable marker for small-cell differentiation in the prostate[24]. However, no data on the frequency of *RB1* and *TP53* mutations in NEPCs and the concordance rate with immunostaining findings are available in TCGA database(**Table 5**)[25].

RB1 and p53 abnormalities are also found in other NE tumors, and the 2022 WHO classification classifies NE tumors as highly differentiated neuroendocrine tumors (NETs) and poorly differentiated neuroendocrine carcinomas (NECs)[26]. NETs are generally classified as G1, G2, or G3 based on their proliferation, whereas NECs are classified as high-grade. In particular, from a pathologist's perspective, there is a need for biomarkers that can distinguish G3 NETs from NECs. In pancreatic cancer, p53 and RB1 are thus incorporated as optional biomarkers, as they emerge as helpful in the differential diagnosis of NETs G3 versus NECs. The IHC patterns for these proteins reflect the underlying molecular changes that seem to be important in the clinical behavior of NEC and in predicting therapy response, as is suggested for tumors with *RB1* mutations, as often seen in SCC[27,28]. IHC is used to detect aberrant expression; however, no previous reports have compared NGS and IHC findings. In addition, although there have been validation studies of *RB1* and *TP53* in *de novo* NEPCs and adenocarcinomas, no studies have compared these findings to t-pure NEPCs. Therefore, it is important to evaluate and compare these factors in this study.

To date, no studies have directly compared IHC findings for RB1 and p53 proteins with genomic analyses of *RB1* and *TP53* alterations in prostate cancer. However, in breast cancer, there has been a report demonstrating frequent co-alterations in the *RB1* and *TP53* genes along with the loss of RB1 and p53 protein expression by IHC, illustrating the utility of combining these two methods[29]. In the current study, the genomic results for *RB1* and *TP53* in cases diagnosed with t-NEPC agreed with the IHC results at a high rate. Concordance rates were 70% (14/ 20 cases) for RB1 and 80% (16/ 20 cases) for p53. A total of 5% (1/20 cases) of RB1 and p53 cases showed no abnormalities in IHC despite the presence of genomic variants. These results indicate that there were no expression abnormalities, and it is possible that the exon mutations captured by NGS were non-functional mutations that affected the protein function but not the expression. To determine whether or not the characteristics of these tumors are

comparable to other RB1-loss NEPCs, it is necessary to compare parameters such as the tumor morphology, genome, and treatment response. However, the small number of patients in this study did not allow us to examine this issue.

In cases presenting with dynamic genomic mutations, such as deletion and frameshift mutations, the results were consistent with a high probability of 91.2% (11/12 cases). In contrast, regarding RB1 IHC, 25% (5/20 cases) had RB1 loss despite the absence of genomic mutations. Epigenetic abnormalities may cause aberrant expression of these genes, and RB1 genomic alterations are enriched in intronic splice-site mutations and structural variants[30]. These may not be detected by targeted sequencing methods, which generally cover exclusively or primarily exonic regions. Therefore, since it is difficult to obtain complete concordance between genome analysis and IHC results, ideally, both tests should be performed to avoid overlooking any abnormal findings. If the concordance rate between the results of both tests is high, it may be advantageous to perform only one of the tests.

The evaluation of RB1 and p53 by IHC may be helpful in the diagnosis of t-NEPC. Due to the limitations of conventional NE markers and morphological evaluation alone, the number of NEPC cases is potentially much more significant. Case 8 had abnormal RB1 and p53 expression in IHC, even though conventional NE markers were negative except for CD56, and the treatment response to EP was good with PR. Three of the four cases with low H-scores for SYN and CgA were also hetero for RB1 IHC but had aberrant p53 expression; it may be essential to evaluate the protein expression of both of these types, rather than either RB1 or p53 alone, to increase sensitivity.

Although IHC and NGS for RB1/*RB1* and p53/*TP53* could not predict the prognosis in this study, it may help diagnose t-NEPC, which is difficult to diagnose using conventional NE markers. In addition, it is tempting to speculate that the inclusion of RB1 and p53 in conventional panels may also help identify clinically significant subgroups of high-grade

metastatic prostate tumors. Although it is not feasible to use both NGS and IHC in all cases due to time and economic costs, if the concordance rate between both is somewhat high, it may be possible to detect many t-NEPC patients at the right time by proactively performing IHC for RB1 and p53, which is less costly. According to the cBioPoral database[31], abnormalities in RB1 and p53 IHC in prostate adenocarcinoma were found in 2.0% and 9.9% of cases, respectively (Table 5). In the current study, 78.9% of t-NEPCs showed abnormalities in RB1 and p53, resulting in a sensitivity of 78.9% and specificity of 98.0% for RB1 and a sensitivity of 78.9% and specificity of 90.1% for p53. In addition, we were able to obtain pathological specimens at the time of the initial prostate cancer diagnosis in 7 of the 20 patients. IHC for RB1 and p53 was performed on these specimens, and interestingly, 42.9% (3/7) had RB1 loss or partial loss, and 35.0% (7/20) had p53 abnormalities before conversion to NEPC (Figure 3). This finding suggests that IHC may be able to detect prostate cancer with a high likelihood of NE transformation. Further studies are needed to accumulate more cases in which IHC evaluated RB1 and p53 from the adenocarcinoma stage and to investigate the usefulness of t-NEPC as an early biomarker.

Several limitations associated with the present study warrant mention. First, the cohort size was small, and this was a retrospective study, which limits the generalizability of the results. Second, there were a few cases in which pathology specimens were unavailable prior to NEPC conversion or IHC of conventional NE markers was not possible. Nevertheless, our findings provide new insights into t-NEPC diagnoses and increase our understanding of the disease process, thereby helping improve the management of t-NEPC.

Conclusion

This study examined the genomic and IHC results of 20 Japanese patients with t-NEPC, with a focus on RB1/*RB1* and p53/*TP53*. Although the utility of RB1 and p53 as biomarkers to predict conversion to t-NEPC and response to platinum drugs remains unclear, an IHC analysis of RB1 and p53 is helpful for making a more accurate diagnosis of t-NEPC. The utility of assessing RB1 and p53 may be demonstrated through the further accumulation of NEPC cases and validation in prostate cancer before NE transformation.

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Figure legends

Figure 1: Concordance between pathology and genomic results

The concordance between NGS and IHC results was high, with 70% (14/20) agreement for RB1/*RB1* and 80% (16/20) for p53/*TP53*.

Figure 2: Progression-free survival stratified by next-generation sequencing and immunohistochemistry findings

Genomic findings tended to be more stratified, but the two groups had no significant differences.

Figure 3: Pathological findings of Case 15

The pathology at the time of the initial diagnosis of prostate adenocarcinoma was already partially lost of RB1 and p53.

Figure 1

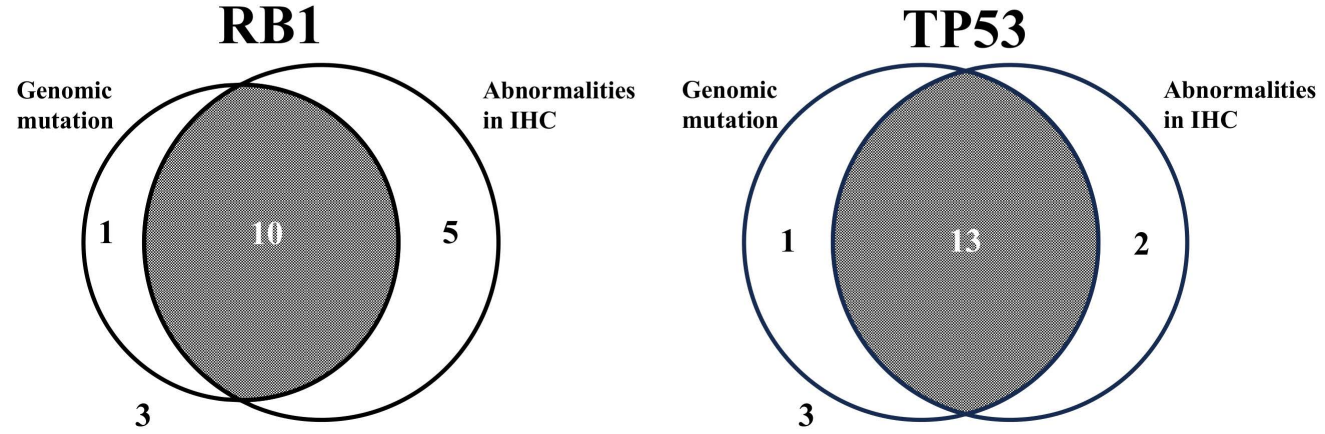
RB1
Genomic mutation
Abnormalities in IHC
1
10
5
3
TP53
Genomic mutation
Abnormalities in IHC
1
13
2
3

Figure 2

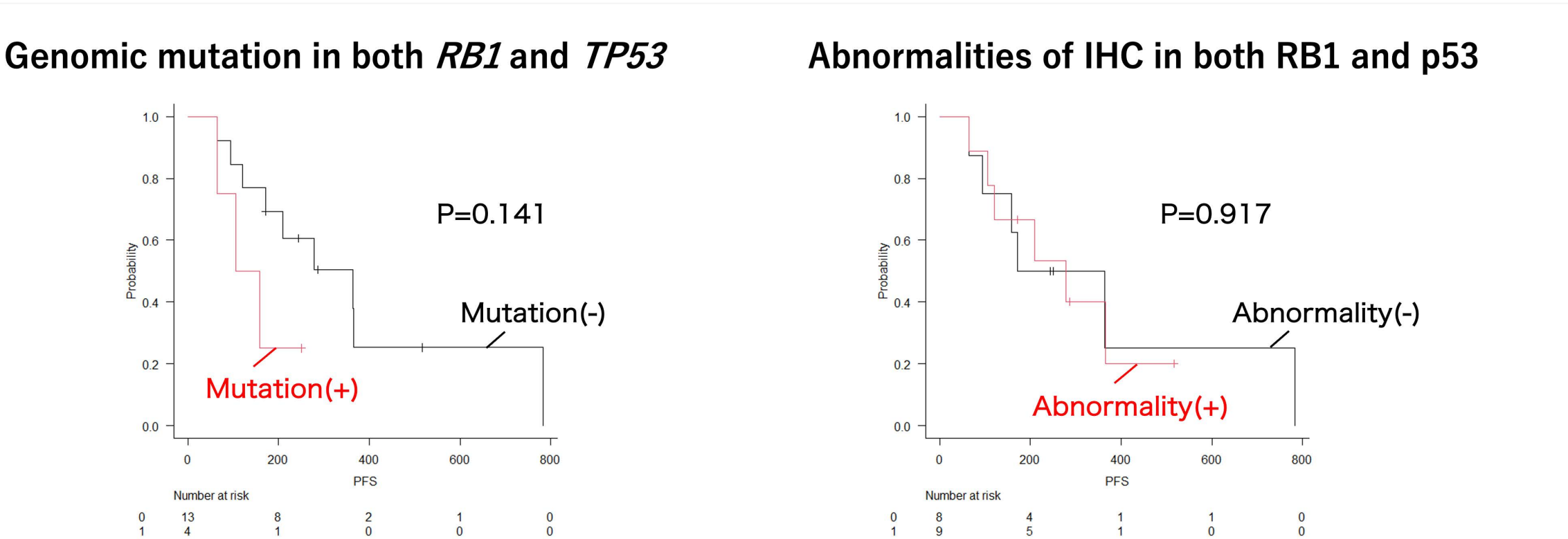
Genomic mutation in both *RB1* and *TP53*
P=0.141
Mutation(-)
Mutation(+)
Probability
1.0
0.8
0.6
0.4
0.2
0.0
0
200
400
600
800
PFS
Number at risk
0 13 8 2 1 0
1 4 1 0 0 0
Abnormalities of IHC in both RB1 and p53
P=0.917
Abnormality(-)
Abnormality(+)
Probability
1.0
0.8
0.6
0.4
0.2
0.0
0
200
400
600
800
PFS
Number at risk
0 8 4 1 1 0
1 9 5 1 0 0

Figure 3

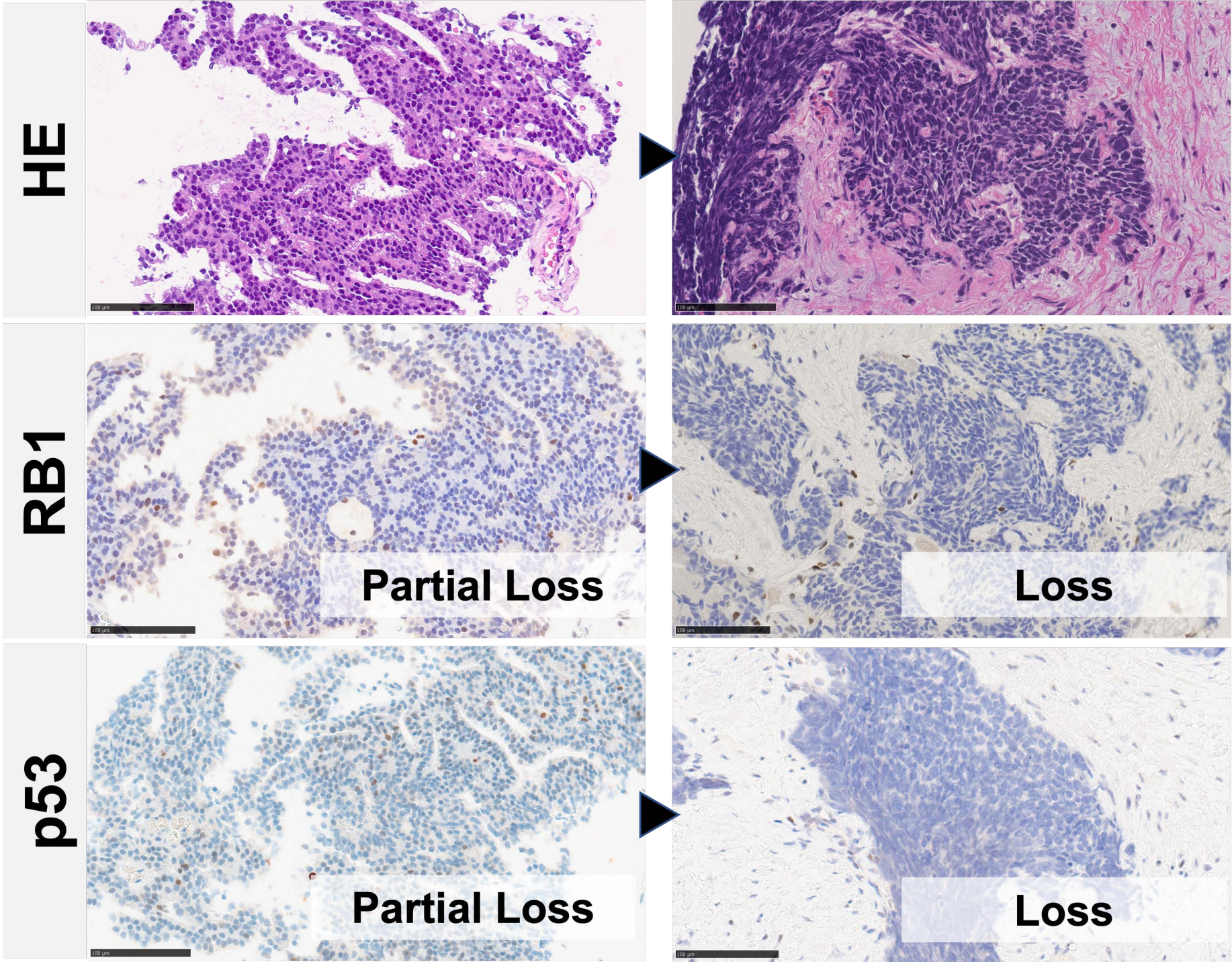

Table 1. Patients characteristics and treatment course before t-NEPC

	Number of patients (%)
Age (yr)	
Median	66.3
Range	49-83
PSA (ng/ml)	
<20	6 (33.3)
20<	12 (66.7)
Gleason score	
< 4+4	2 (10.0)
4+4 ≤	18 (90.0)
Metastasis status	
M0	7 (35.0)
M1	13 (65.0)
Metastatic region	
Bone	11 (55.0)
Lung	3 (15.0)
Liver	1 (5.0)
Local therapy	
Prostatectomy	3 (15.0)
Radiation	3 (15.0)
None	14 (70.0)
Kind of hormonal treatment	
Only CAB	11 (55.0)
Only ARSI	3 (15.0)
CAB + ARSI	6 (30.0)

CAB, combined androgen blockade, ARSI, androgen receptor signaling inhibitor

Table 2. Caracteristics of neuroendocrine prostate cancer patients

	Number of patients (%)
Age (yr)	
Median	70.9
Range	51-83
Metastasis status	
M0	4 (20.0)
M1	16 (80.0)
Metastatic region	
Bone	12 (60.0)
Lung	8 (40.0)
Liver	7 (35.0)
Biopsy site	
Prostate	7 (35.0)
Lymph node	5 (25.0)
Liver	4 (20.0)
Bone	2 (10.0)
Bladder	1 (5.0)
Subcutaneous	1 (5.0)
PSA (ng/ml)	
< 4.0	14 (70.0)
4.0 ≤	6 (30.0)
Synaptophysin, IHC	
Positive	14 (70.0)
Negative	1 (5.0)
N/A	5 (25.0)
ChromograninA, IHC	
Positive	9 (45.0)
Negative	7 (35.0)
N/A	4 (20.0)
CD56, IHC	
Positive	8 (40.0)
Negative	0

N/A	12 (60.0)
Time to NEPC, months	
< 24	7
24 ≤	13

IHC, Immunohistochemstry

Table 3. IHC and NGS findings for RB1/*RB1* and p53/*TP53* in 20 Japanese patients of t-NEPC.

***RB1*/RB1**		**IHC**			
		Loss	**Wild**	**N/A**	**Total**
Genomic status	**Deletion**	**4**	**0**		**4**
	Frameshift	**4**	**0**		**4**
	Splicesite	**2**	**1**		**3**
	Normal	**5**	**3**	**1**	**9**
		15	**4**	**1**	**20**

***TP53*/p53**		**IHC**				
		Loss	**Hetero**	**Over expression**	**N/A**	**Total**
Genomic status	**Deletion**	**1**	**0**	**0**		**1**
	Frameshift	**1**	**0**	**2**		**3**
	Nonsense	**3**	**0**	**2**	**1**	**6**
	Missense	**1**	**1**	**2**		**4**
	Splicesite	**1**	**0**	**0**		**1**
	Normal	**1**	**3**	**1**		**5**
		8	**4**	**7**	**1**	**20**

IHC, Immunohistochemistry

NGS, Next-generation sequencing

Table 4. NGS and IHC findings for each patient.

	RB1			TP53			NE marker (t-NEPC)				Biopsy site (t-NEPC)
	Adeno	t-NEPC		Adeno	t-NEPC		SYN	CgA	CD56	INSM1	
Case	IHC	IHC	Genomic mutation	IHC	IHC	Genomic mutation	IHC	IHC	IHC	IHC	
1	N/A	Loss	Splice site	N/A	Loss	None	250	80	300	50	Lymph node
2	Retain	Loss	Deletion	Loss	Loss	Splice site	280	250	300	200	Liver
3	N/A	Loss	Frameshift	N/A	Loss	Nan-sense	280	10	300	280	Bladder
4	Retain	Loss	Deletion	Over expression	Over expression	Frameshift	290	10	300	130	Liver
5	N/A	Hetero	None	N/A	Hetero	None	280	80	200	20	Bone
6	Retain	Loss	Frameshift	Over expression	Over expression	Nan-sense	280	40	300	270	Lymph node
7	N/A	Loss	Deletion	N/A	Hetero	None	300	0	100	80	Liver
8	Partial loss	Loss	None	Over expression	Over expression	Frameshift	0	0	180	60	Prostate
9	Retain	Loss	Deletion	Over expression	Over expression	None	250	0	80	200	Liver
10	N/A	Loss	Frameshift	N/A	Loss	Nan-sense	200	150	N/A	80	Prostate
11	N/A	N/A	None	N/A	N/A	Nan-sense	N/A	N/A	N/A	N/A	Bone
12	N/A	Loss	None	N/A	Loss	Deletion	280	250	300	130	Subcutaneous
13	N/A	Loss	Splice site	N/A	Hetero	None	280	0	280	200	Lymph node
14	Loss	Loss	None	Over expression	Over expression	Nan-sense	100	30	300	160	Prostate
15	Partial loss	Loss	None	Partial loss	Loss	Nan-sense	270	100	290	150	Prostate
16	N/A	Hetero	Splice site	N/A	Loss	Frameshift	50	10	10	0	Prostate
17	N/A	Loss	Frameshift	N/A	Hetero	Miss-sense	N/A	N/A	N/A	N/A	Prostate
18	N/A	Hetero	None	N/A	Over expression	Miss-sense	10	0	5	0	Lymph node
19	N/A	Loss	None	N/A	Loss	Miss-sense	250	5	300	150	Lymph node
20	N/A	Hetero	None	N/A	Over expression	Miss-sense	20	10	250	0	Prostate

*H-score

Table. 5 Validation data for RB1/*RB1* and p53/*TP53* in prostate adenocarcinoma and CRPC.

Abnormalities in IHC

	Prostatic adenocarcinoma	mCRPC	t-NEPC (Current study)
RB1	**2%***	**-**	**78.9%**
p53	**9.9%***	**-**	**78.9%**

Genomic mutation

	Prostatic adenocarcinoma	mCRPC	t-NEPC (Current study)
RB1	**0.81%****	**3.45%****	**55.0%**
TP53	**10.9%****	**30.1%****	**70.0%**

*cBioPortal for Cancer Genomics

**The Cancer Genome Atlas Program

IHC, Immunohistochemistry

mCRPC, metastatic castration resistant prostate cancer

t-NEPC, treatment-related neuroendocrine prostate cancer