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High Rate of BRAF and RET/PTC Dual Mutations Associated with Recurrent Papillary Thyroid Carcinoma

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Abstract

Purpose—Papillary thyroid carcinoma (PTC), the most common thyroid malignancy, usually possesses BRAF mutation or rearranged in translation (RET)/PTC rearrangements. PTC usually possesses BRAF mutation or RET/PTC rearrangements. The mutation status of patients with recurrent PTC has never been characterized in a large population.

Experimental Design—Mutation status was determined in a cohort of 54 patients with recurrent PTC and analyzed for clinicopathologic relationships. BRAF and ras mutations were determined by PCR and sequencing of genomic DNA. RET/PTC rearrangements were analyzed by reverse transcription-PCR.

Results—BRAF mutation in exon 15 (V600E) was found in 42/54 (77.8%) recurrent PTC patients. The RET/PTC rearrangements were detected in 9 of 54 (16.7%) patients. In addition, 5 of 54 (9.3%) recurrent PTC patients had both a BRAF mutation and a RET/PTC rearrangement. The prevalence of tumors with dual mutations found in the recurrent population far exceeds the frequency historically reported for patients with primary PTC. Patients with dual mutations were significantly older (80% older than 45 years) than patients with a BRAF mutation alone (38% older than 45 years).

Conclusions—Recurrent PTC is significantly associated with a predominant BRAF mutation. RET/PTC rearrangements, although commonly associated with primary PTCs in younger patients, are uncommonly found in recurrent PTC patients. In addition, the incidence of dual mutations was higher in patients with recurrent PTC than in those primary PTC, as reported by others.

Papillary thyroid carcinoma (PTC) is the most common type of thyroid malignancy accounting for 70% to 80% of thyroid cancer cases (1). In most cases, PTC has a very good

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No potential conflicts of interest were disclosed.

prognosis, particularly in patients younger than ages 45 years at the time of diagnosis. However, roughly 5.7% of patients at 5 years and 9.4% of patients at 10 years with primary PTC will experience a recurrence of tumor (2). Recurrence typically occurs in the neck region, either in lymph nodes or in the thyroid bed, and less commonly in distance sites such as lung or bone. Patients who were older than 45 years at the time of initial diagnosis have a much worse prognosis when the cancer recurs. The 15-year mortality rate for this group is ~30% for patients with local recurrence in the neck, and roughly 50% for patients with recurrence at distance sites (1,3,4).

Several types of genetic alterations have been found in primary PTC, including mutations in BRAF or ras, and rearrangements of the RET or NTRK1 tyrosine kinase receptor. The incidence of BRAF mutations ranges from 29% to 83% depending on the cohort studied (5). The most common type of BRAF mutation found in primary PTC is a T to A substitution at nucleotide 1799 in exon 15, which results in conversion of a valine to glutamic acid at codon 600 (V600E) of the BRAF protein (6,7). BRAF mutation has been associated with poor prognosis in PTC patients (8–10). The incidence of RET/PTC rearrangements in PTC ranges from 2.5% to 67.0% depending on the cohort studied (11,12). Most patients with RET/PTC rearrangements in their primary PTCs were younger than 45 years (13–17). A total of 11 different RET/PTC rearrangements have been reported; the most widely studied are RET/PTC1 (RET kinase fused with the *H4* gene; ref. 18), RET/PTC2 (RET kinase fused with the regulatory subunit RI α of cyclic AMP-dependent protein kinase A; ref. 19), and RET/PTC3 (RET kinase fused with the *RFG* gene; ref. 20). Mutations in ras and NTRK1 rearrangements have been reported but are very rare in primary PTC (21–26).

The mutation profile of patients with recurrent PTC is largely uncharacterized. In this study, we analyzed the mutation status of 54 patients with recurrent PTC and analyzed the data by patient age, race, gender, and tumor stages. We found that recurrent PTC is significantly associated with a predominant BRAF mutation. RET/PTC rearrangements, although commonly associated with primary PTCs in patients younger than 45 years, are uncommonly found in recurrent PTC patients. In addition, we found that 9.3% of these recurrent tumors had acquired both a BRAF mutation and a RET/PTC rearrangement. Patients with dual mutations were older and had more advanced tumors than patients with a BRAF mutation alone. These data suggest that recurrent disease may be associated with more than one mutation and that recurrent PTC is likely a genetic selection event.

Materials and Methods

Thyroid samples

Archival formalin-fixed and paraffin-embedded papillary thyroid cancer specimens (all recurrences were from lymph node metastases) were examined in 68 consecutive patients who underwent surgery for recurrent PTC between September 1, 2003, and August 31, 2004, and were retrieved retrospectively from the Department of Pathology at The University of Texas M. D. Anderson Cancer Center. After examining the H&E staining, those patients (total 54 patients) containing >70% tumor cells in total cellularity were selected for this study. In addition, the primary tumors (formalin-fixed and paraffin-embedded thyroid specimens) from five patients were also used in this study in an effort to compare to their delayed metastatic lymph nodes. Tumor samples were obtained in accordance with protocols approved by the institutional review board. Tumors were classified according to the histopathologic typing of the WHO (27,28).

DNA extraction

A 10- μ m section was taken from each paraffin block and subjected to deparaffinization twice in xylene for 15 min at 55°C. After two 15-min periods of rehydration in 100% ethanol, the tissue pellets were dried and digested with proteinase K (0.5 mg/mL; Ambion) at 55°C for 3 d. Fresh proteinase K was added to the samples everyday. After extraction with phenol/chloroform, genomic DNA was precipitated and 100 ng of DNA was used in PCR.

RNA extraction

Total RNA was extracted from two 10- μ m sections of paraffin-embedded tumor tissue using an Optimum FFPE RNA isolation kit (Ambion) according to the manufacturer's protocols. Two micrograms or less of total RNA were reverse transcribed (RT) by Superscript II (Invitrogen Corp.) in a 25- μ L total reaction volume containing RT buffer, random hexamers (Invitrogen), deoxynucleotide triphosphate, and RNase inhibitor (Roche Applied Science).

Detection of BRAF and ras mutations

Exons 11 and 15 of BRAF and codons 12/13 and 61 of N-ras, K-ras, or H-ras were analyzed by PCR and then sequenced at the DNA Sequencing Core Facility at M. D. Anderson Cancer Center. The primers for these BRAF exons and ras genes (synthesized by Sigma-Aldrich) were published previously and are summarized in Table 1 (13,29,30). Genomic DNA (100 ng) was used as a template in a 50- μ L PCR mixture containing 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L MgCl₂, 0.2 mmol/L deoxynucleotide triphosphates, and 1 U of advantage HF-2 (Clontech). Cycling conditions were as follows: initial denaturation (95°C for 2 min) and then 35 cycles (denaturation at 94°C for 30 s, annealing at 52–62°C for 30 s, and synthesis at 68°C for 30 s), followed by a final extension of 3 min at 68°C. All PCR products were visualized by electrophoresis on a 2% agarose gel and purified by using a QIAEX II gel extraction kit (QIAGEN). The positive control for BRAF mutation was a PTC cell line (NPA87), which was kindly provided by Dr. Jerome Hershman (Veterans Affairs Greater Los Angeles Healthcare System, Los Angeles, CA).

Detection of RET/PTC rearrangements

Two sets of PCR primers (primers A–D and nested primers; Table 1) were synthesized by Integrated DNA Technologies, Inc., to detect the RET/PTC1 (primers A and D, and nested A), RET/PTC2 (primers B and D, and nested B), and RET/PTC3 (primers C and D, and nested C) rearrangements (13,31). For PCR, 1 μ L of RT mixture was amplified with 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L MgCl₂, 0.2 mmol/L deoxynucleotide triphosphates, and 1.25 U of AmpliTaq Gold DNA polymerase (Applied Biosystems). Cycling conditions were as follows: initial denaturation (95°C for 9 min) and then 35 cycles (denaturation at 95°C for 30 s, annealing at 53–58°C for 30 s, and synthesis at 72°C for 30 sec), followed by a final extension of 7 min at 72°C. All PCR products were visualized by electrophoresis on a 2% agarose gel, and gel images were documented by Gel Doc XR (Bio-Rad). The positive control for RET/PTC1 was TPC-1, a PTC cell line obtained from Dr. Jerome Hershman (Veterans Affairs Greater Los Angeles Healthcare System, Los Angeles, CA). Plasmids containing the *RET/PTC2* or *RET/PTC3* cDNA were kindly provided by Dr. Sissy Jhiang (The Ohio State University, Columbus, OH) and were used as positive controls for RET/PTC2 and RET/PTC3, respectively. The negative control for the RET/PTC1 rearrangement was NPA87.

Statistical analysis

Standard χ^2 test and Student's *t* test were used. A *P* value of 0.05 or less was considered significant.

Results

Mutation status

To study the mutation status of recurrent PTC, we prepared genomic DNA and total RNA from the tumors of 54 patients who underwent surgery for recurrent disease at M. D. Anderson Cancer Center in the 1-year period beginning in September 2003.

Nested RT-PCR was used to detect the RET/PTC1, RET/PTC2, and RET/PTC3 rearrangements, which were identified in 9 of 54 samples (16.7%; Fig. 1A and B). The rearrangements included 1 sample with the RET/PTC1 rearrangement (Fig. 1A), 4 with the RET/PTC2 rearrangement (Fig. 1B), and 4 with the RET/PTC3 rearrangement (Fig. 1B).

To detect BRAF mutations, both exons 11 and 15 of BRAF were sequenced. A total of 42 of 54 samples (77.8%) showed a heterogeneous T to A substitution at nucleotide 1799 of BRAF and only the patients with dual mutation were shown (Fig. 1C). BRAF mutation was confirmed by sequencing both strands of DNA. No mutation in exon 11 of BRAF was detected in any of the samples.

Of all the samples containing RET/PTC rearrangements or a BRAF mutation, 5 samples (9.3%) contained both a BRAF mutation and a RET/PTC rearrangement (Fig. 1). To get an understanding of the molecular changes happening in these tumors with dual mutations, the mutation status in primary tumor specimen was also examined. We found BRAF mutation in all 5 primary tumors and no RET/PTC rearrangements were detected by nested RT-PCR (data not shown).

Mutations could not be detected in 8 of 54 samples. These samples were further tested for H-ras, N-ras, and K-ras (codons 12/13 and 61) mutations using genomic DNA and NTRK1 mutation using total RNA (data not shown). The only mutation found was a K-ras mutation at codon 61 (1 sample), resulting in conversion of a glutamine to lysine. Five of the 7 samples had a well-known silent single nucleotide polymorphism at nucleotide 81 (codon 27) of H-ras, changing T to C, but no other mutations were detected.

Correlation between mutation status and clinicopathologic variables

Base on their mutation status, this cohort of 54 patients with recurrent PTC was separated into 5 groups: BRAF mutation only, RET/PTC rearrangements only, BRAF mutation and RET/PTC rearrangement dual mutations, K-ras mutation only, or no detectable mutation (none). Patient's gender and race of each group are listed in Table 2, and age makeup of each group is shown in Fig. 2. The stages of the primary PTC for these patients are listed in Table 3. All patients have received radio iodine treatment. Histologic features and stages of each patient's recurrent carcinoma, along with the number of positive lymph nodes harvested and confirmed by a pathologist, and time span between primary tumor and recurrent tumor are listed in Table 4. The majority of patients in this cohort were Caucasian (Table 2), had no evidence of distance metastasis (M0; 98%; Table 4), and had metastasized to lymph nodes (N1; 63%; Table 3).

In the BRAF mutation only group (total 37 patients or 68.5% of all recurrent PTC patients), the majority of the patients were women (70.3%; Table 2) and younger than 45 years (62.2%; $P < 0.001$; Table 2; Fig. 2). In these patients with BRAF mutation only, most of their tumors were in T2 and T4 stages (37.8% in T2 and 27% in T4 Table 3). The time span between primary and recurrent PTC was between 0.75 and 15 years (Table 4).

Most patients with the RET/PTC rearrangements only (total 4 patients or 7.4% of all recurrent PTC patients) were men (75%; Table 2). In this group, all the tumors were T1 and

T2 stages and no tumors were T3 or T4 stages (Table 3). The time span between primary and recurrent PTC was between 0.5 to 8 years (Table 4).

In the group of the patients with both a BRAF mutation and a RET/PTC rearrangement (total 5 patients or 9.3% of all recurrent PTC patients), patients were older (80% older than 45 years; Fig. 2). These patients had more advanced tumors (80% in T4) than those patients with BRAF mutation only (27% in T4; Table 3).

Discussion

The mutation status of patients with recurrent PTC has never been characterized in a large population. In this study, we focused on a specific group of thyroid carcinomas, recurrent PTC, and analyzed mutation status from a cohort of 54 consecutive patients from whom specimens were collected at M. D. Anderson Cancer Center between September 1, 2003, and August 31, 2004.

Most mutational studies on PTC have been done on primary disease. Only a few reports have mentioned recurrent PTC (32–34). Nakayama et al. (32) reported 14 cases of recurrent PTC, of which 85.7% had a BRAF mutation. The ages of these 14 patients were not mentioned in their study (32). In our cohort, BRAF mutation (V600E) was detected in 77.8% of tumor samples. We can conclude, therefore, that this is the major mutation in recurrent PTC. In fact, BRAF has become a diagnosis and, by some, a prognostic marker for primary PTC. BRAF mutation has been associated with poor outcomes in differentiated and/or undifferentiated cancers derived from primary PTC (5,7,9,35–37). BRAF mutation has also been linked with advanced tumor stages (T3/T4 versus T1/T2) in primary PTC (8,9,38). However, in recurrent PTC, tumors with BRAF mutation only were evenly distributed between T3/T4 and T1/T2 stages (37.8% versus 40.5%, respectively).

The incidence of RET/PTC rearrangements in primary PTC is lower than that of BRAF mutation depending on the cohort studied (11,12). Recent reviews on primary PTC have indicated that the frequency of RET/PTC rearrangements in PTC is at 20% (39,40). We have also observed a low incidence (16.7%) of RET/PTC rearrangements in our recurrent PTC cohort. All three types of RET/PTC rearrangements (RET/PTC1, RET/PTC2, and RET/PTC3) were detected in recurrent PTC as well as in primary PTC as reported by others (11,12). Several methods have been described for the detection of RET/PTC rearrangements, including nested RT-PCR, hybridizing RT-PCR products with an internal probe, Southern blotting, real-time RT-PCR, and fluorescence *in situ* hybridization (11–13,41). The most sensitive method of all is the hybridization after RT-PCR with an internal probe, which can detect 1.5 to 5 cells with RET/PTC rearrangements, but the rearrangement cannot be confirmed by any other methods (41). With nested RT-PCR being the most popular method of choice, all the other methods confirm the results obtained by nested RT-PCR. We choose the nested RT-PCR method to detect all of the RET/PTC rearrangements in our study based on the fact that it is a simple and reliable method. However, we cannot exclude that RET/PTC rearrangements other than RET/PTC1, RET/PTC2, and RET/PTC3 exist in recurrent PTC population.

There are conflicting reports regarding the relationship of patient age and tumor stage in cases of primary PTC with BRAF mutation (8,9,32,36,38,42). BRAF mutation in most primary PTC has been associated with greater patient age (32,36). This is not the case in our cohort of recurrent PTC; most patients with BRAF mutation only (62.2%) were younger than 45 years. For patients younger than 45 years, RET/PTC rearrangements have been the most common type of the mutation in primary PTC (13–17). In our cohort of the recurrent PTC patients, RET/PTC rearrangements are uncommonly found (16.7% of total patients).

These data suggested that recurrent PTC is likely a genetic selection event. Further studies with more patient samples are needed to confirm our findings and determine whether additional molecular events produce an increasing rate of recurrent PTC.

In most reported cases of PTC, BRAF mutation and RET/PTC rearrangements are considered as separated events in PTC without overlap (37,43,44). Recently, Zhu et al. (41) reported that both BRAF mutation and RET/PTC rearrangements were detected in the same primary PTC from 3 patients, in which RET/PTC rearrangements took place in <10% of tumor cells examined. Using immunohistochemistry, Xu et al. (45) detected increased expression of RET in a BRAF-mutated tumor. Of 54 recurrent PTC tested in this study, 5 (9.3%) had both mutations. Although the number of the patients with the dual mutations was lower than the numbers of patients with BRAF mutation only, it was observed in a much higher percentage than in all the primary PTC cases reported thus far. In addition, patients with the dual mutations are older and have more advanced tumors, with 80% having originally presented with T4 primary tumors. The methods we used to detect these mutations cannot distinguish whether the mutations were in the same cells or in different cells. After examining the primary tumors from the patients with dual mutations, we found that only one type of mutation (BRAF mutation) was present in the primary tumors. These data suggest that these patients acquired one mutation first for their primary PTC and then developed a second mutation during the course of their disease. In addition to BRAF mutation and RET/PTC rearrangement, ras mutations are rare in primary PTC. Ras mutations are found in only 10% to 30% of primary PTC (39,40,42,46). We only found one recurrent PTC specimen containing a K-ras mutation.

Seven samples in our cohort had no detectable mutation in any genes analyzed. Whether these represent “false negative” or true negative is not known at this time. Several factors may contribute to the lower detection sensitivity (47). First, only 3 of 11 RET/PTC rearrangements were tested, although the other 8 RET/PTC rearrangements are rare. The inconsistent quality of RNA from paraffin-embedded tissues and unavailability of snap-frozen tissues from these patients were the main impediments to detection of RET/PTC rearrangements and NTRK1 rearrangements because detection of both rearrangements requires total RNA (48). Lastly, there are always potential limitations on the sensitivity of the detection method used.

In conclusion, our study provides basic information about the mutation status of recurrent PTC. The incidence and prevalence of PTC continues to increase, and a greater knowledge of the molecular development and regulation of these neoplasms is required if we are to advance our understanding and develop more therapies for these tumors.

Translational Relevance

This study analyzed the mutation status of 54 patients with recurrent papillary thyroid carcinoma (PTC). This type of analysis has never been characterized in a large population for recurrent PTC. This study illustrates not only that BRAF mutation was detected in 77.8% of recurrent PTC but also that 9.3% of tumors contained dual mutations (a BRAF mutation and a RET/PTC rearrangement). Despite all the mutations published in the literature, rarely are dual mutations found in primary PTC. It has been accepted by others that only one mutation is needed to develop primary PTC. In addition, 62% of recurrent PTC patients younger than 45 years had BRAF mutation, whereas RET/PTC rearrangements are unquestionably the most common mutations in young patients with primary PTC. Our finding of simultaneous mutations suggested that the mechanism

for developing more aggressive recurrent PTC may be different from that the more favorable well-differentiated primary PTC.

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Fig. 1.

Detection of RET/PTC rearrangements and BRAF mutation in recurrent PTC. *A*, RET/PTC1 rearrangement from patient #39 was detected in total RNA. The positive control (+control) was cDNA made from TPC-1 cells (positive for RET/PTC1 rearrangement), and negative control (-control) was cDNA made from NPA87 cells (BRAF mutated and no RET/PTC rearrangements). *B*, total RNA was prepared from paraffin-embedded tissues (from patients #10, #18, #25, #28, #42, #47, #48, and #57) and RT-PCR was done to detect RET/PTC 2 or RET/PTC3 rearrangement (arrow). The PCR product was visualized on a 2% agarose gel stained with ethidium bromide. Actin was used as a control. The positive controls for RET/PTC2 and RET/PTC3 were plasmids containing the cDNAs of each gene and negative controls were buffer only without any cDNA added. *C*, PCR product of exon 15 of BRAF from patients with dual mutations (#18, #25, #28, #42, and #57) was sequenced with forward primer (*left*) and reverse primer (*right*). Arrows, the transition from T to A by forward primer and A to T by reverse primer.

Fig. 2.

Age of recurrent PTC patients in each mutation group. Fifty-four recurrent PTC patients were separated into five groups by their mutation status, including BRAF mutation only (*BRAF*), RET/PTC rearrangements only (*RET/PTC*), dual mutations with a BRAF mutation and a RET/PTC rearrangement (*B & R*), K-ras mutation only (*K-ras*), or no detectable mutation (*none*). *Solid line*, age at 45 y as discussed in the Results. The graph was generated by Prism software.

Table 1

PCR primers used in this study

	Forward primer (5'→3')	Reverse primer (5'→3')	Size (bp)	Anneal T°C
BRAF exon11	TCCCTCTCAGGCATAAGGTAA	CGAACAGTGAATATTTCTTTGAT	313	58
BRAF exon15	TCATAATGCTTGCTCTGATAGGA	GGCCAAAAATTTAATCAGTGGA	224	58
Primer A+ D	ATTGTCATCTCGCCGTTC	CTTTCAGCATCTTCACGG	306	55
Primer B+D	GAGGGAGCTTTGGAGAAC	CTTTCAGCATCTTCACGG	266	55
Primer C+D	AAGCAAACCTGCCAGTGG	CTTTCAGCATCTTCACGG	240	55
Nested A	ACAAACTGAAGTGCAAGGCA	GCCTTGACCACTACTTTTCCAAA	151	55
Nested B	CTTTGATTTATGGAACACCG	ACTTTTCCAAATTCGCCTTC	175	53
Nested C	CCTGCCAGTGGTTATCAAGC	GGCCACCGTGGTGTACCCTG	219	58
Actin	CCTCCCTGGAGAAGAGCTACGAG	GACAGCACTGTGTTGGCGTACAG	199	62
H-ras codon 12/13	ATGACGGAATATAAGCTGGT	CCTTATAGTGGGGTCGTATT	110	52
H-ras codon 61	AGGTGGTCATTGATGGGGAG	AGGAAGCCCTCCCCGGTGCG	109	62
K-ras codon 12/13	GGCCTGCTGAAAATGACTGAA	GGTCCTGCACCAGTAATATGC	166	62
K-ras codon 61	CAGGATTCTACAGGAAGCAAGTAG	CACAAAGAAAGCCCTCCCCA	135	62
N-ras codon 12/13	ATGACTGAGTACAACTGGT	CTCTATGGTGGGATCATATT	111	52
N-ras codon 61	TCTTACAGAAAACAAGTGGT	GTAGAGGTTAATATCCGCAA	174	62

Table 2

Patients mutation status associated with gender and race

Mutations	BRAF	RET/PTC	BRAF & RET/PTC	K-ras	None
Patients					
Total numbers	37	4	5	1	7
Gender					
Female	26	1	2	1	5
Male	11	3	3	0	2
Race					
Caucasian	26	4	4	0	5
Hispanic	6	0	1	1	1
Asian	4	0	0	0	0
Black	0	0	0	0	1
Unknown	1	0	0	0	0

Table 3
Patients' mutation status associated with clinicopathologic variables in primary PTC

Stage grouping	BRAF*	RET/PTC*	BRAF & RET/PTC*	K-ras	None*
Age <45 y					
Stage I	23 (62.2)	2 (50.0)	1 (20.0)	0	4 (57.1)
Stage II	0	0	0	0	0
Stage III	0	0	0	0	0
Stage IV	0	0	0	0	0
Unknown	1	0	0	0	0
Age ≥45 y					
Stage I	1 (2.7)	0	0	0	0
Stage II	2 (5.4)	0	1 (20.0)	0	2 (28.6)
Stage III	8 (21.6)	1 (25.0)	2 (40.0)	1	1 (14.3)
Stage IV	0	0	1 (20.0)	0	0
Unknown	2	1	0	0	0
T stage					
T1	1 (2.7)	1 (25.0)	1 (20.0)	0	0
T2	14 (37.8)	2 (50.0)	0	0	2
T3	4 (10.8)	0	0	0	2
T4	10 (27.0)	0	4 (80.0)	0	2
Unknown	8	1	0	1	1
N stage					
N0	9 (24.3)	0	1 (20.0)	0	3
N1	22 (59.5)	3 (75.0)	4 (80.0)	1	4
Unknown	6	1	0	0	0

* Numbers in parentheses represent percentage in that type of mutation.

Table 4
Patients' mutation status associated with clinicopathologic variables in recurrent PTC

Mutations	BRAF	RET/PTC	BRAF & RET/PTC	K-ras	None
Number of nodes positive					
0-1	3	1	0	0	0
2-9	21	1	4	1	7
10-19	13	1	0	0	0
>20	0	1	1	0	0
Histologic subtype					
Papillary	37	4	4	1	6
Follicular	0	0	1	0	0
Tall cell	0	0	0	0	1
M stage					
M0	37	4	4	1	7
M1	0	0	1	0	0
Time to recurrent/persistent					
Years	0.75-15	0.5-8	0.33-5	4	0.25-7