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Genetic polymorphisms of enzymes related to oral tegafur/uracil therapeutic efficacy in patients with hepatocellular carcinoma

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Abstract

Background
Recently, oral tegafur/uracil therapy in patients with hepatocellular carcinoma (HCC) has not been a lot less administration, only a single agent. But this treatment for HCC has not been studied much that it is related to the 5-fluorouracil (5-FU) metabolic enzymes until now. We investigated genetic polymorphisms of the 5-FU metabolic enzymes in Japanese patients with HCC.

Methods
In 58 Japanese hepatitis C virus-seropositive HCC patients, we examined two genetic polymorphisms of the metabolic enzymes: cytochrome P450 2A6 (CYP2A6) and dihydropyrimidine dehydrogenase (DPD). As an efficacy check, we investigated genetic polymorphisms of the variable number of tandem repeat (VNTR) of thymidylate synthase (TS) and classified the genotypes into high (low response to 5-FU treatment) or low (high response to 5-FU treatment) expression type.

Results
The frequency of CYP2A6*4 allele (no-activity allele) among 58 HCC patients was 0.233, and homozygous genotype (*4/*4) was found in 5 patients. Heterozygous genotype (T/C) of DYPD*9 (T85C) was detected in 8 patients, and the frequency of DYPD*9 allele among 58 HCC patients was 0.069. Of 58 patients, 42 were classified as high expression type and 16 as low expression type for TS VNTR. Fifteen of these 16 patients appeared to have normal CYP2A6 metabolic activity, and 13 of these 15
patients probably had normal DPD metabolic activity.

Conclusions

Only 13 of 58 HCC patients (22.4%) may be responded effectively in treatment of oral tegafur/uracil. Therefore, when administering oral 5-FU in patients with HCC, it is important to consider three genetic polymorphisms (CYP2A6, DPYD and TS) of the metabolic enzymes.

Keywords

hepatocellular carcinoma, tegafur/uracil, metabolic enzyme, genetic polymorphism, therapeutic efficacy

Background

Advanced hepatocellular carcinoma (HCC) with portal venous invasion, lymph nodes metastasis and/or extrahepatic metastasis (such as bone or lung metastasis) has very poor prognosis, and systemic and/or hepatic arterial infusions chemotherapy is the main approach to treatment [1-7]. Many regimens of systemic and/or arterial infusions chemotherapy for such disease condition have been reported, such as intraarterial cisplatin /systemic interferon-alpha , systemic oral tegafur/uracil, intraarterial cisplatin, systemic 5-fluorouracil (5-FU) /mitoxantrone /cisplatin, systemic cisplatin/interferon α -2b/doxorubicin/fluorouracil, systemic doxorubicin/cisplatin/capecitabine , intraarterial 5FU/ systemic interferon-alpha [1-7]. Recently, sorafenib, a oral multi-kinase inhibitor
with anti-angiogenic activity, was approved for the treatment of advanced HCC [8-9]. But the median overall survival in patients with advanced HCC is less than 12 months, even if they underwent these treatments [1-9]. Because of the poor prognosis, besides efficacy of chemotherapy, maintenance of the patient’s quality of life is another important consideration. Systemic oral tegafur/uracil therapy is often done with fewer side effects than any other treatment [1-9]. Therefore, oral tegafur/uracil is an essential agent for the treatment of advanced HCC. In such clinical settings, when oral tegafur/uracil therapy is started, the following three genetic predisposed factors should be considered: 1) whether oral tegafur/uracil is steadily bioactivated in the liver; 2) whether activated plasma 5-FU concentration is adequate but not unacceptably toxic; and 3) whether target enzyme of 5-FU in the patient is sensitive to the administered 5-FU.

First, an important factor is bioactivation of orally administered tegafur/uracil which itself is a prodrug. Tegafur/uracil is absorbed from the gastrointestinal tract and is transported to the liver via the portal circulation [10]. And then, it is activated to 5-FU by the hepatic enzyme cytochrome P450 2A6 (CYP2A6) [11,12]. In Japanese, mutations that abolish or lower the enzyme activity of CYP2A6 have been reported; they were CYP2A6*4 (deletion type) and CYP2A6*9 (-48T>G) with allele frequencies of 19.0~20.1% and 19.0~21.3%, respectively [13-15]. In these individuals, orally administered tegafur/uracil may not be adequately bioactivated.

Second, current data have demonstrated that 5-FU plasma levels can vary several folds despite equal doses administered to cancer patients [16]. A wide inter-individual variation of plasma 5-FU level has been demonstrated even after
optimizing doses by body surface area [16]. One of the factors that generate such wide variation may be genetic polymorphisms of the 5-FU metabolic enzyme dihydropyrimidine dehydrogenase (DPD) in individual patients. DPD is a primary and rate-limiting enzyme in pyrimidine base catabolism, and it is also known to be responsible for the metabolic degradation of 5-FU [17-20]. Since more than 80% of administered 5-FU is degraded in vivo by DPD to fluorinated β-alanine, the level of DPD catalytic activity affects the efficacy or toxicity of 5-FU [16-20]. DPD deficiency has been reported to be associated with unacceptable toxicity of 5-FU based chemotherapy in cancer patients. Further, homozygous carriers of DPYD, which are mutated alleles of DPD, exhibit more severe toxic responses to 5-FU treatment than individuals with wild type alleles. At present, the association between genetic polymorphism of DPYD and DPD activity has been reported [17-24]. Among Japanese, mutations resulting in no or decreased DPD activity: namely, DPYD*3, *9AB, *10, *11, and *12, should be checked when oral tegafur/uracil is administered, even though that are present in very small populations [21-24].

On the other hands, the target enzyme for 5-FU is thymidylate synthase (TS). The TS gene (TYMS) is known to be polymorphic, having either double (2R) or triple tandem (3R) repeats (variable number of tandem repeat; VNTR) of 28 base pair sequence in the promoter region [25-29]. TS expression predicts response to 5-FU based chemotherapy, and the expression seems to be determined by the TYMS promoter gene. Using additional functional single nucleotide polymorphism (SNP) of the TYMS VNTR (G/C), Kawakami et al. reported that TYMS genotypes can be classified into high expression and low expression types, which indicate the TS translation rate [29]. They also demonstrated that colon cancer patients classified as low expression type have
longer survival rate following 5-FU-based chemotherapy compared with patients not treated with 5-FU based chemotherapy, and suggested that it is clinically important to examine polymorphisms of \textit{TYMS} \cite{29}.

5-FU based chemotherapy for HCC has not been studied much that it is related to the 5-FU metabolic enzymes until now. In the present study, we analyzed the genetic polymorphisms of \textit{CYP2A6} and \textit{DPYD} in HCC patients, as well as examined SNP of \textit{TYMS} VNTR and classified them into high and low expression types, with the aim to investigate the proportion of Japanese HCC patients who would potentially benefit from 5-FU based chemotherapy.

\section*{Methods}

\subsection*{Subjects and DNA samples}

Fifty-eight Japanese cirrhotic patients with HCC were studied. Cirrhosis and HCC were diagnosed by liver biopsy or computed tomography and ultrasonography. Patient characteristics are summarized in Table 1. The use of patient blood samples for this study had been approved by Jikei University Ethics Committee. Peripheral blood was collected after obtaining written informed consent. These DNA samples had been obtained from January, 2005 to February, 2009. The samples were numbered, unlinked, and tested anonymously. DNA was extracted from peripheral blood mononuclear cells by the standard protocol. DNA samples were also obtained from 44 Japanese healthy volunteers (aged 20 to 60 years) to determine the frequency of \textit{TSER} (thymidylate synthase enhancer region) in control subjects. The volunteers were assessed to be
physically normal based on a comprehensive health examination.//

**Analysis of CYP2A6**

*CYP2A6*4 is an allele of gene defect and loss of function. PCR was performed to detect the *CYP2A6*4 using primers B4 and UTRAS1. The PCR reaction mixture contained 10× buffer, 2 mM dNTPs, 10 pmol of each primer, 0.2 µl of Ampli Taq Gold and 100 ng of genomic DNA. Amplification was performed by 35 cycles of denaturing at 94°C for 1 minute, annealing at 58°C for 1 minute, and extension at 72°C for 1 minute. After amplification of a 1.32 kb fragment, digestion using two restriction enzymes; ACCII-, and Eco81I, was carried out. After overnight digestion at 37°C, the samples were subjected to electrophoresis on a 2% TAE gel. The ACCII-RFLP products of 1040 and 280 bp, and Eco81I-RFLP products of 728 and 428 bp were identified as the homozygous mutant allele of *CYP2A6*4.

**Genetic analysis of CYP2A6 (CYP2A6*4 (deletion) Invader® copy number assay)**

We used the quantitative capability of the Invader® system to determine gene copy number by comparing the target gene signal (*CYP2A6*) with that of a reference gene such as α-actin. The *CYP2A6* probe set was designed to react specifically with only the *CYP2A6* sequence (Table 2). The relative ratios of the *CYP2A6* and reference gene signals from each assay allowed us to identify and quantitate the deletion alleles of *CYP2A6*.

Genomic DNA was extracted using the method given above. Invader® reactions were performed using 384-well plates with reagents containing Cleavase® XI enzyme for genomic DNA, and both FAM and RED FRET cassettes. In brief, 3µL of
pre-denatured DNA samples (20 ng/μL) or no target control (10 ng/μL tRNA) were added to the appropriate wells followed by addition of 1.2 μL of signal probes/Invader® oligo Mix, 1.4 μL of FRET Mix and 0.4 μL of Cleavase/ MgCl2 solution for Genomic DNA and an overlay of 6 μL of molecular biology-grade mineral oil. Following reagent dispensing, plates were spun for 10 seconds at 1000 rpm (120×g), incubated at 63°C for 3 hours in a PTC-100™ thermal cycler, and then directly read with a CytoFlour 4000 fluorescence plate reader using the settings given above [30]. For the genotype determination, Fold Over Zero (FOZ) is used to confirm the validity of each assay of the samples [12]. The value was calculated by the program provided by Third Wave Technologies. For the copy number assay, the NET FAM and RED FOZ values (for CYP2A6 and α-actin) were calculated and the ratio of the CYP2A6 to α-actin NET FOZ was calculated to identify CYP2A6 copy number [30, 31].

For genotype determination, Fold Over Zero (FOZ) is used to confirm the validity of each assay (Neville M et al., 2002). The value was calculated by the software provided by Third Wave Technologies. For the copy number assay, the NET FAM and RED FOZ values (for CYP2A6 and α-actin, respectively) were calculated and the ratio of CYP2A6 to α-actin NET FOZ was calculated to identify CYP2A6 copy number (Neville M et al., 2002).

**DPYD analysis**

*DPYD* SNP analysis was performed by the Invader® system to determine gene copy number by comparing the target gene signals [*DPYD*3 (C189 deletion), *9 (T85C), *10 (G2983T), *11 (G1003T), and *12 (G62A)] with that of the reference gene α-actin. Invader® reactions were performed by the same conditions as described above.
**TS genotyping**

TS genotyping was performed by PCR for VNTR using the template of genomic DNA, according to the methods described by Kawakami et al. [29]. The amplified DNA fragments were analyzed by electrophoresis on a 4% agarose gel followed by staining with ethidium bromide.

For heteroduplex analysis, PCR was performed using a primer labeled with fluorescein 5-isothiocyanate and analyzed by Spreadex gel. The PCR product from a sample of 2R/3R genotype forms a heteroduplex that appears on electrophoresis as bands at positions different from the 3R band. The heteroduplex products showed 3 different electrophoresis patterns. PCR was performed using forward primer TS25 AGGCGCGCGGAAGGGGTCTT and reverse primer TS18 TCCGAGCCGGCCACAGGCAT. The PCR conditions were the same as those for TS VNTR. The PCR product was digested with HaeIII followed by electrophoresis in 4% agarose gel and ethidium bromide staining. Analysis was performed at least twice to confirm the genotype. According to the study by Kawakami et al., TS genotypes of 2R/3G, 3C/3G, and 3G/3G were considered high expression (H) type, while 2R/2R, 2R/3C or 3C/3C were considered low expression (L) type [29].

**Statistical analysis**

Chi-square test was used to compare the allele frequencies of all genotypes studied and frequencies of altered metabolic phenotypes between patients and healthy subjects. A p value < 0.05 was considered statistically significant.
Results

The results of genetic analyses conducted in this study are summarized in Table 2.

Genetic analysis of CYP2A6

The frequency of \textit{CYP2A6}*4 allele among 58 HCC patients was 0.233. The genotypes with respect to CYP2A6*4 in 58 patients were as follows: wild type (wt/wt) in 36 patients, heterozygous type (*4/wt) in 17, and homozygous type (*4/*4) in 5.

\textit{DPYD} analysis

Among 58 HCC patients, we did not find any SNP allele of *3 (C1897deletion), *10 (G2983T), *11 (G1003T), or *12 (G62A), but detected heterozygous mutation (T/C) of \textit{DPYD}*9 (T85C) in 8 patients. The frequency of \textit{DPYD}*9 allele in 58 HCC patients was 0.069. The homozygous mutation (C/C) of \textit{DPYD}*9 was not found in 58 patients.

\textit{TYMS} genotyping

In 58 HCC patients, the \textit{TYMS} allele frequencies in terms of G>C SNP in VNTR were as follows: 0.095 for 2R, 0.471 for 3G, and 0.431 for 3C. The frequencies in 44 control subjects were 0.159 for 2R, 0.420 for 3G, 0.409 for 3C, and 0.011 for 5R. There was no significant difference in the allele frequency between HCC patients and healthy subjects (odds ratio: 1.567). According to the classification of high or low
expression group reported by Kawakami, et al. 〔29〕. 42 of 58 HCC patients was classified as high expression type (2R/3G, 3C/3G, 3G/3G) and 16 of 58 patients as low expression type (2R/2R, 2R/3C, 3C/3C). Among 44 healthy subjects, 28 were classified as high expression type and 16 as low expression type. No significant difference was detected between two groups (odds: 0.667).

Discussion

Recently, oral tegafur/uracil therapy in patients with hepatocellular carcinoma (HCC) has not been a lot less administration, only a single agent. But adjuvant therapy with oral tegafur/uracil for patients with advanced HCC is necessary to decrease the rate of recurrence, prolong the disease-free period, and maintain the quality of life for such patients. However, most physicians are not concerned as to whether the orally administered tegafur/uracil enters the systemic circulation and reaches the target site adequately and appropriately without rising to a toxic concentration. Before initiating treatment with oral tegafur/uracil, at least two genetic polymorphisms should be checked: CYP2A6 by which tegafur is activated to 5-FU in the liver and DPD by which 5-FU is metabolized to the inactive form in the liver.

In Japanese, among the reported genetic polymorphisms that lead to loss of CYP2A6 metabolic activities, CYP2A6*4 is the most frequent allele detected 〔13-15〕. In present study, the allele frequency of CYP2A6*4 was 0.233, which is not significantly difference from that of 444 healthy Japanese subjects (0.184) reported by Ariyoshi et al. 〔32〕. Five of 58 HCC patents was homozygous for CYP2A6*4
resulting in no enzyme activity, with a frequency of 0.086. This frequency is also not significantly different from that in healthy Japanese (0.034).

A number of studies have reported substantial inter-individual variability of plasma 5-FU levels [16,33]. Although therapeutic drug monitoring (TDM) is an essential tool in such situation, an easy-to-use TDM for 5-FU is yet not available in the clinical setting. Therefore, physicians are required to take great care to avoid the unacceptable toxicity when treating with fluorouracil agents without TDM. Furthermore, the variability of plasma 5-FU level is known to be mainly due to the variability of the activity of the key enzyme DPD, and a linear relationship has been demonstrated between DPD activity and 5-FU plasma clearance [34,35]. The activity of DPD varies as a result of DYPD genetic polymorphism [17-19,21-35]. In the present study, we investigated five DYPD genetic polymorphisms in 58 HCC patients and found only one SNP of DYPD*9AB (T85C). The genotype frequency of DYPD*9AB polymorphism in Japanese has been reported previously: the C-allele frequency is 3.7 to 6.3% [21-24], which is almost similar to the frequency (8 of 116 alleles, 6.9%) in 58 HCC patients. A study of 300 Taiwanese subjects has reported a C-allele frequency of 4.3%, and no CC homozygous genotype [36]. We also detected no CC genotype in the present study. However, a German study of 157 Caucasians has demonstrated a higher frequency of 19.4% [37,38], indicating that the DYPD 85 C allele is more frequently observed in Caucasians than in Orientals.

DYPD*9AB (T85C) polymorphism, in which cysteine without electron polarity at the 29th amino acid is substituted by arginine with electron polarity, might result in a difference in enzyme activity. Some gene expression experiments using E. coli showed that the enzyme activity of DPD encoded by the 85 C allele was too low to be detected
In a study on TC heterozygous genotype cancer patients, DPD activity measured by HPLC was more than 30% lower than that of normal subjects \[23\]. However it remains controversial whether all individuals with TC genotype have markedly low DPD activity \textit{in vivo}.

On the other hand, in the setting of treating cancer patients with fluorouracil, beside plasma levels of 5-FU, physician should also consider whether 5-FU works ultimately as an anticancer drug in individual cancer patient. The target enzyme for 5-FU is TS and the levels of TS expression in target tumors possibly determine the sensitivity to 5-FU as a TS inhibitor. TS mRNA is known to have a unique tandem repeat sequence in the 5’UTR and is polymorphic in terms of the number of this repeat (VNTR) \[25\text{-}28\]. The repeat length controls TS protein expression. Further, Kawakami et al. demonstrated additional polymorphism in VNTR of the TS gene as SNP \[29\]; G/C polymorphism in TS VNTR, which is also a genetic variation that can potentially predict the effectiveness of 5-FU \[29\]. They also reported that TS VNTR is related to TS protein expression by affecting the translational activity of TS mRNA, and TS alleles show frequent loss of heterozygosity. In their analysis of TS VNTR in which a heteroduplex formed between 2R- and 3R-derived PCR products was separated by high-resolution gel, they observed different electrophoresis patterns of the heteroduplex. They confirmed the presence of G/C polymorphic change (SNP) and classified the polymorphic allele as *2G, *2C, *3G and *3C in accordance with the combination of the SNP and VNTR \[29\]. Functional analysis showed that the plasmid construct with 3G sequence had 3–4 times greater efficiency of translation than the other polymorphic sequences. Then they analyzed the TS genotype according to the combination of the novel SNP and VNTR, in patients with resected colorectal cancer (64 patients treated
with 4 different oral 5-FU derivatives as adjuvant therapy and 47 patients with no adjuvant treatment). The genotypes were classified into a high expression type (*2/*3G, *3C/*3G and *3G/*3G) and a low expression type (*2/*2, *2/*3C and *3C/*3C). This new genotype classification was used to predict survival in the 111 patients. For the low expression type, patients who received oral fluoropyrimidine survived longer than the patients with no treatment. For the high expression type, however, no benefit of oral fluoropyrimidine was observed [29].

Using the classification proposed by Kawakami et al., we analyzed 2 polymorphisms of TS VNTR and classified the genotypes into high or low expression type in 58 HCC patients. Only 16 of 58 HCC patients had the low expression type, who would possibly respond to 5-FU treatment. Investigators have discussed whether the proposed classification of high or low expression type is appropriate to predict 5-FU anticancer efficacy. Many subsequent studies have supported this classification [39-42], but some others have not [43,44]. Even though this issue remains controversial, TS genotyping may be a reliable tool to predict 5-FU clinical efficacy.

For a physician who faces the decision of initiating oral tegafur/uracil chemotherapy in a cancer patient, it is essential to consider whether this drug will reach the cancer cells in a therapeutic amount and is effective to eliminate these cells. In our present study examining three potential genetic barriers (CYP2A6, DPDY and TS) in 58 HCC patients, 16 HCC patients (No1-No16 at Table2) were classified as low TS expression type and would possibly respond to 5-FU treatment. Fifteen (No1-No15 at Table2) of these 16 patients appeared to have normal CYP2A6 metabolic activity, and 13 (No3-No15 at Table2) of these 15 patients probably had normal DPD metabolic activity. Hypothetically, only 13 of 58 HCC patients (22.4%) treated optimally with 5-FU are
likely to have favorable therapeutic outcome. Future, 5-FU based chemotherapy in patients with advanced HCC is great potential to contribute to the response and survival rate in the single agent or multiple agents, to investigate three genetic polymorphisms (CYP2A6, DPYD and TS) of the 5-FU metabolic enzymes.

Conclusions

The current study reveals important role three genetic polymorphisms (CYP2A6, DPYD and TS) of the 5-FU metabolic enzymes on 5-FU based chemotherapy in patients with HCC. Therefore, when administering oral 5-FU in patients with HCC, it is important to consider genetic polymorphisms of the metabolic enzymes, and tailor-made treatment in each case.

Abbreviations

HCC, hepatocellular carcinoma; 5-FU, 5-fluorouracil; CYP2A6, cytochrome P450 2A6; DPD, dihydropyrimidine dehydrogenase; TS, thymidylate synthase; VNTR, variable number of tandem repeat; TYMS, thymidylate synthase gene; SNP, single nucleotide polymorphism; TSER, thymidylate synthase enhancer region; TDM, therapeutic drug monitoring.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions

NF and AO conceived the study design. NF, IT, HN, SA, and AO contributed to patient recruitment and carried out clinical study procedures. NF, SA and AO analyzed and interpreted the data. NF and AO prepared the manuscript. NF and AO revised the manuscript critically. All authors read and approved the final manuscript.
Table 1: Patient characteristics

<table>
<thead>
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<th>M / F</th>
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<tr>
<td>Gender</td>
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</tr>
<tr>
<td>Age</td>
<td>years old</td>
<td>70 ± 7</td>
</tr>
<tr>
<td>Clinical stage (T.N.M)</td>
<td>I / II / III / IVa / IVb</td>
<td>11 / 29 / 15 / 2 / 1</td>
</tr>
<tr>
<td>Child-Pugh</td>
<td>A / B / C</td>
<td>48 / 7 / 3</td>
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<tr>
<td>AST</td>
<td>(IU/L)</td>
<td>74 ± 31</td>
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<tr>
<td>ALT</td>
<td>(IU/L)</td>
<td>68 ± 62</td>
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<tr>
<td>Total protein</td>
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<td>7.6 ± 0.8</td>
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<tr>
<td>Albumin</td>
<td>(g/dl)</td>
<td>3.7 ± 0.5</td>
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<tr>
<td>Total bilirubin</td>
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<td>1.3 ± 2.2</td>
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<tr>
<td>Prothrombin index</td>
<td>(%)</td>
<td>79 ± 12</td>
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</tbody>
</table>

AST, aspartate aminotransferase; ALT, alanine aminotransferase. Normal ranges (Central Chemical Laboratory, Daisan Hospital, The Jikei University School of Medicine, Tokyo): AST 5-28 IU/l; ALT 5-35 IU/l; total protein 6.8-8.3 g/dl; albumin 3.5-5.2 g/dl; total bilirubin 0.2-0.8 mg/dl; prothrombin index 80-100% (obtained by dividing the prothrombin time of the patient by the reference value of control subjects).
Table 2  Three genetic polymorphisms in 58 patients with hepatocellular carcinoma

<table>
<thead>
<tr>
<th>No.</th>
<th>CYP2A6*4 Genotype</th>
<th>DPYD*9 (T85C)</th>
<th>G&gt;C SNP in TYMS</th>
<th>No.</th>
<th>CYP2A6*4 Genotype</th>
<th>DPYD*9 (T85C)</th>
<th>G&gt;C SNP in TYMS</th>
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<td>1</td>
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<td>3C/3C</td>
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<td>T/T</td>
<td>3G/3C</td>
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<td>T/T</td>
<td>3G/3C</td>
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<td>34</td>
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<td>T/T</td>
<td>3C/3C</td>
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<td>T/T</td>
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<td>T/T</td>
<td>3C/3C</td>
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<td>15</td>
<td>Wt / Wt</td>
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<td>16</td>
<td>*4 / *4</td>
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