The relative protein abundance of UGT1A alternative splice variants as a key determinant of glucuronidation activity *in vitro*.

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Running Title: Modulation of UGT activity by i2 protein content in cells.

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Abbreviations: UGT, UDP-glucuronosyltransferases; i1, isoform 1; i2, isoform 2; co-IP, co-immunoprecipiation; UDPGA, Uridine diphospho-glucuronic acid; ER, endoplasmic reticulum; SDS-PAGE, sodium dodecyl sulfate poly-acrylamide gel electrophoresis; SN-38, 7-Ethyl-10-hydroxycamptothecin.

ABSTRACT

Alternative splicing (AS) is one of the most significant components of the functional complexity of human UDP-glucuronosyltransferase enzymes (UGTs), particularly for the *UGT1A* gene which represents one of the best example of a drug metabolizing gene regulated by AS. Shorter UGT1A isoforms i2 are deficient in glucuronic acid transferase activity but function as negative regulators of enzyme activity through protein-protein interaction. Their abundance, relative to active UGT1A enzymes, is expected to be determinant of global transferase activity of cells and tissues. Here, we tested whether the i2-mediated inhibition increases with greater abundance of the i2 protein relative to the i1 enzyme using the extrahepatic UGT1A7 as a model, and a series of 23 HEK293 clonal cell lines expressing variable content of i1 and i2 proteins. Upon normalization for i1, a significant reduction of SN-38 glucuronide formation was observed for i1+i2 clones (mean of 53%) compared to the reference i1 cell line. In these clones, the i2 protein content greatly varied (38% to 263% relative to i1) and exposed 2 groups; 17 clones with i2<i1 (60%±3%) and 6 clones with i2≥i1 (153%±24%). The inhibition induced by i2 was more substantial for clones displaying i2≥i1 (74.5%; p=0.001) compared to those with i2<i1 (45.5%). Co-immunoprecipitation supports a more substantial i1-i2 complex formation when i2 exceeds i1. We conclude that the relative abundance of regulatory i2 proteins has the potential to drastically alter the local drug metabolism in the cells, particularly when i2 surpasses the protein content of i1.

INTRODUCTION

Alternative splicing (AS) of pre-mRNA is a common process affecting most human genes and effectively increasing the coding potential of the genome and allowing the synthesis of several structurally and functionally distinct protein isoforms. One of the best example of drug metabolizing genes regulated by AS are the UGT1A family members encoded by a single gene locus on chromosome 2q37.1. This is made possible by the presence of 13 mutually exclusive first exons, which are followed by a shared genomic region including four other common exons. The *UGT1A* locus is also subjected to AS at its 3'UTR end with alternative terminal exons 5 leading to a new class of UGT1As and nine shorter protein isoforms named i2, that are deficient in glucuronic acid transferase activity (Girard et al., 2007). These non-conjugating i2 proteins act as natural modulators of UGT1A-mediated glucuronidation (Girard et al., 2007; Bellemare et al., 2010a; Bellemare et al., 2010b; Bellemare et al., 2010c). They are widely co-expressed in most healthy human tissue structures, consistent with a regulatory function mediated through direct protein-protein interaction between regulatory i2 proteins and i1 enzymes (Bellemare et al., 2010a; Bellemare et al., 2010b; Bellemare et al., 2011). It is thus plausible that AS plays a significant role in regulating the enzyme activity level and that the abundance of enzymatically inactive i2 proteins can alter the local drug metabolism in the cells. To gain potential insights into this hypothesis, we tested if the relative abundance of regulatory i2 proteins controls cellular UGT activity by using a heterologous human embryonic kidney 293 cell system to produced clonal cell lines that express variable amount i1 and i2 proteins of the extrahepatic UGT1A7 as a model. The enzyme activity mediated by this UGT1A was largely repressed in the presence of its i2 homolog in co-expression experiments (~80%) (Bellemare et al., 2010b). We also assessed if the abundance of i2 protein content in cells influences the extent of i1-i2 associated complexes by co-immunoprecipitation (co-IP).

MATERIAL AND METHODS

Cell culture, expression systems and microsomal fractions. Human embryonic kidney HEK293 cells were obtained from the American Type Culture Collection (distributed by Cedarlane, Burlington, Ontario, Canada) and cultured as specified. HEK293 cells were transfected with pcDNA3.1A/UGT1A7_i1-His-Myc and pcDNA6A/UGT1A7_i2-V5-His expression plasmids using Lipofectin (Life Technologies, Ontario, Canada). Resistance to geneticin (G418) (1 mg/mL) and blasticidine (10 µg/mL) (Wisent, St-Bruno, Qc, Canada) was used to select for potential positive clones. A total of 23 clones expressing both UGT1A7 isoforms (i1+i2) were isolated and confirmed for expression of both i1 and i2 isoforms as specified below. Microsomal fractions were prepared with cells disrupted using 3 X 10 seconds of sonication and centrifuged twice at 12 200 *g* for 22 minutes. The membrane fractions were then collected by sedimentation of the supernatant at 105 000 *g* for 2 hours. Microsomal fractions were resuspended in microsomal buffer (2.62 mM KH_2PO_4, 1.38 mM K_2HPO_4, 20% glycerol, pH 7.0, 0.5 mM DTT). The protein concentrations were determined using BCA protein assay (Fisher Scientific, Ottawa, Ontario, Canada).

Enzymatic assays and protein expression. Enzymatic assays were performed as reported using 100 µM SN-38 incubated for 3 hours at 37°C, glucuronide formation was measured by mass spectrometry (Bellemare et al., 2010b). The enzyme activity is reported as relative glucuronidation rates (pmol/min/mg protein) expressed as the mean of at least two independent experiments performed in triplicate and normalized for UGT protein content (i1 active enzyme) assessed by Western blot analysis. Student's *t* test (2 sided) was used to evaluate statistical significance of the correlation or to compare glucuronidation rates and isoforms expression between the groups (i2 < i1 and i2 ≥ i1) (significantly different at $p \leq 0.05$). The presence of

UGT1A7 proteins was assessed by Western blot analysis by resolving 20 µg of microsomal fractions on 10% SDS-PAGE gel and proteins were transferred onto nitrocellulose membranes using Trans-Blot Turbo Transfer System (Bio-Rad, Ontario, Canada). A primary polyclonal anti-human UGT1A RC-71 (1:1000) (Gagne et al., 2002) and a secondary donkey anti-rabbit IgG polyclonal antibody, horseradish peroxidase-conjugated ($1:1X10^4$) were used (GE Healthcare, Québec, Canada). The immunoreactive bands were visualized using the Western Lightning Plus-ECL chemiluminescence substrate (Perkin Elmer, Ontario, Canada) with Fuji Medical X-Ray Film (Christie InnoMed Inc., Québec, Canada). The relative levels of UGT proteins were estimated by integrated optical density using the ImageJ program (NIH).

Co-immunoprecipitation assays. Selected HEK293-UGT1A7 i1+i2 clonal cells ($10x10^6$ cells) were lysed for 45 minutes on ice with 1 ml of lysis buffer (0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl, 0.3% deoxycholic acid, 1% Igepal, 1 mM EDTA, complete protease inhibitor (Roche Applied Science, Laval, Qc, Canada)). Lysates were homogenized by pipetting up and down through fine needles (18G followed by 20G) 10 times on ice, then centrifuged for 15 min at 13 000 *g* and the supernatant was collected. Then, 1 mg of pre-cleared lysate (cell lysate incubated at 4°C for 30 minutes with protein G sepharose beads) was combined with 1 µg of anti-myc monoclonal antibody (Sigma, ON, Canada) 1 hour at 4°C and beads were added for overnight incubation at 4°C to immunoprecipitate protein complexes as previously described (Bellemare et al., 2010b). Immunoprecipitated proteins were resolved on 10% SDS-PAGE and blotted membranes were probed with a specific anti-v5 monoclonal antibody linked with horseradish peroxidase (1:5000) (Invitrogen, ON, Canada).

RESULTS AND DISCUSSION

The cellular model based on human HEK293 cells (UGT negative) was used to constitutively express both UGT1A7 isoforms i1 and i2, and established 23 cellular clones (i1+i2). Individual clones were tested for glucuronidation activity with a high affinity substrate SN-38, and analyzed by Western blotting to ascertain relative protein content of i1 and i2 (Fig.1A). The i2 protein was expressed in all 23 clones at a level varying from 38% to 263% relative to i1. Upon normalization for the content of the i1 enzyme, we observed a significant reduction in the rates of SN-38G formation for all clones co-expressing i1+i2 compared to the reference cell line expressing only the i1 enzyme (Fig.1B). The overall mean inhibition of SN-38G formation was of 53% with notable variability (from 2 to 87% inhibition of glucuronidation activity). This is consistent with our previous reports exposing that i2 isoforms have a repressive effect on rates of formation of glucuronide products from several endogenous and exogenous substrates, when i2 are co-expressed with their cognate i1 isoforms in HEK293 cells (e.g., i1 and i2 from UGTs 1A1, 1A6, 1A7, 1A8 and 1A9) (Girard et al., 2007; Bellemare et al., 2010a; Bellemare et al., 2010b). Active UGT1As_i1 and inactive UGT1As_i2 isoforms were shown to be co-expressed in human tissues including liver, kidney, stomach, intestine, and colon (Bellemare et al., 2011), further supporting a dominant-negative role of UGT1As_i2. In addition, their regulatory function is sustained by results of siRNA-mediated depletion of endogenous i2 in two human colon cancer cellular models that lead to increased cellular UGT activity (Bellemare et al., 2010c). An observation that was recently replicated using the hepatocellular carcinoma HepG2 cell line, which displays slightly higher mRNA transcript levels of UGT1A1_v2/v3 transcripts compared to UGT1A1_v1 (Jones et al., 2012), although protein levels were not measured. Based on the inhibitory role of UGT1A_i2 isoforms, we might expect that i2-mediated inhibition increases

with i2 protein abundance and lead to a more significant repression in cells displaying i2 protein content superior to i1. Pearson's coefficient correlation was 0.25 between the expression ratios of UGT1A splice forms and the percent inhibition of enzyme activity ($p=3.35x10^{-8}$). A closer analysis of the relative expression of UGT1A7 isoforms in clonal cell lines revealed two groups based on the ratio of enzymatically inactive i2 and active i1. Indeed, 17 clones expressed i2 in a proportion less than 50% of the active i1 (i2 < i1; 60% ± 3%), while 6 clones showed an expression of i2 equal or superior to i1 (i2 ≥ i1; 153% ± 24%). Consistent with the hypothesis, a more dramatic repression of SN-38 glucuronidation activity of 74.5% (p=0.001) was observed for clones displaying i2 ≥ i1 compared to clones with i2 < i1 (45.5%) (Fig.2A). In our previous work, UGT1A_i2 variants were shown to form hetero-oligomers with active UGT1A_i1 enzymes leading to inactive i1-i2 complexes and likely explaining the reduced enzyme activity (Bellemare et al., 2010a; Bellemare et al., 2010b). We further observed a broad diversity of hetero-oligomeric complexes between every UGT1As_i1 and i2, raising the possibility that any given i2 could potentially interact with whichever active i1 in the ER membrane *in vivo*, although this remains to be demonstrated. The relative abundance of active/inactive complexes, either in the form of dimers or higher oligomers, would then be determinant of global transferase activity of the cell. In line with this postulate, we used co-IP to better appreciate the intermolecular interactions between i1 and i2 proteins in four selected clones with similar amount of i1 but variable i2 protein content (either higher or lower than i1). A stronger signal is observed from immunoprecipitates obtained with clones presenting superior content of inactive i2 relative to active i1 (130% and 170% of i1) compared to clones with less i2 expression (50% and 70% of i1) (Fig.2B). Since all clones presented comparable i1 protein content, the amount of

complexes is likely a direct reflection of the intensity of the immunoprecipitates, and does not reflect variable levels of i1 expression in the protein preparations.

Overall, findings support that when the amount of inactive i2 exceeds the amount active i1, formation of i1-i2 inactive complexes is more substantial and would result in greater inhibition of transferase activity. It is likely that this observation applies to all UGT1A since we previously reported that along with a significant inhibition of i1 activity by i2, i1 isoform of each UGT1A co-immunoprecipitated its respective i2 homolog as well as all other i2s, indicating that they can form heteromeric complexes (Bellemare et al., 2010a). The relative abundance of UGT1A splice variants at the protein level in human tissues remains largely unknown and it is thus difficult to extrapolate our findings from *in vitro* data to the *in vivo* situation. Nevertheless, immunohistochemistry experiments using a specific anti-i2 polyclonal antibody support that i2 proteins are widely expressed in human tissues, most often concurrently with i1, and that expression pattern generally depends on tissue structures (Bellemare et al., 2011). Although the i2 protein levels were <10% of the i1 protein levels in few healthy human liver samples that we previously studied, a predominance of the i2 protein during tumorigenesis was noted (Bellemare et al., 2011) and is corroborated by the mRNA expression patterns recently reported for the hepatocarcinoma HepG2 cells (Jones et al., 2012).

It is plausible that depending on the tissue structures, cell types and disease states, that the expression pattern of i2 proteins becomes predominant relative to i1 and thus, could have profound implications for overall UGT activity. Besides, the degree of inhibition observed with clones having less i2 than i1 is also meaningful and quite comparable to the effect of some genetic variants. Additional studies are warranted to better characterize the expression patterns of UGT1A splice variants and should help to elucidate their function *in vivo*.

AUTHORSHIP CONTRIBUTIONS

Participated in research design: **Rouleau and Guillemette.**

Conducted experiments: **Roberge, Falardeau, Rouleau and Villeneuve.**

Performed data analysis: **Rouleau and Guillemette.**

Wrote or contributed to the writing of the manuscript: **Rouleau and Guillemette.**

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FOOTNOTES

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FIGURES LEGENDS

Figure 1. HEK293-UGT1A7 clonal cell lines stably expressing i1/myc and i2/v5 were generated (i1+i2; n=23) and compared to a reference cell line expressing only the UGT1A7 enzyme (i1). (A) Protein content was initially assessed for each clone (20 ug of microsomes) by Western blotting using an in-house anti-UGT1A polyclonal antibody (RC-71; 1:1000). A reference cell line co-expressing i1+i2 was also loaded onto each gel as a positive control (+). Upper bands (~58 kDa) represent i1 while lower bands (~48 kDa) represent i2. (B) Enzymatic assay were performed in triplicate with 100 µM of SN-38 for 3h. SN-38G was quantified by MS. Rates of formation of SN-38G (mean ± SEM) from two independent experiments are expressed as pmol/min/mg protein and normalized for i1 enzyme expression. Data are presented according to the relative level of i2 expression with i1 as the reference cell line (no i2 expression), i1+i2 clonal cells displaying either i2 < i1 or i2 ≥ i1.

Figure 2. (A) Relationship between UGT activity and i2 expression level relative to i1, for 17 i1+i2 clonal cells displaying less i2 relative to i1 (i2 < i1) and compared to 6 clones with greater content of i2 relative to i1 (i2 ≥ i1). Percentage of inhibition for each category is indicated as a mean ± SEM and *P*-values (*t* test, 2 sided). (B) Results of co-IP assays for a subset of four clonal cell lines displaying similar amount of i1, but different i2 protein content; 2 clones with i2 < i1 vs. 2 clones with i2 ≥ i1. Relative protein content was assessed using an anti-UGT1A polyclonal antibody and percentages of i2 relative to i1 are indicated for each clone in the lower panel (referred to as expression). Upper panel represent co-IP experiments where i1/myc was immunoprecipitated (polyclonal anti-myc, Sigma, ON, Canada) and the interacting protein i2/v5 was revealed (anti-v5-hrp, 1:5000, Life Technologies, ON, Canada). The UGT1A7 cell line expressing only i1 is used as a negative control and i2 could not be revealed upon precipitation.

Figure 1

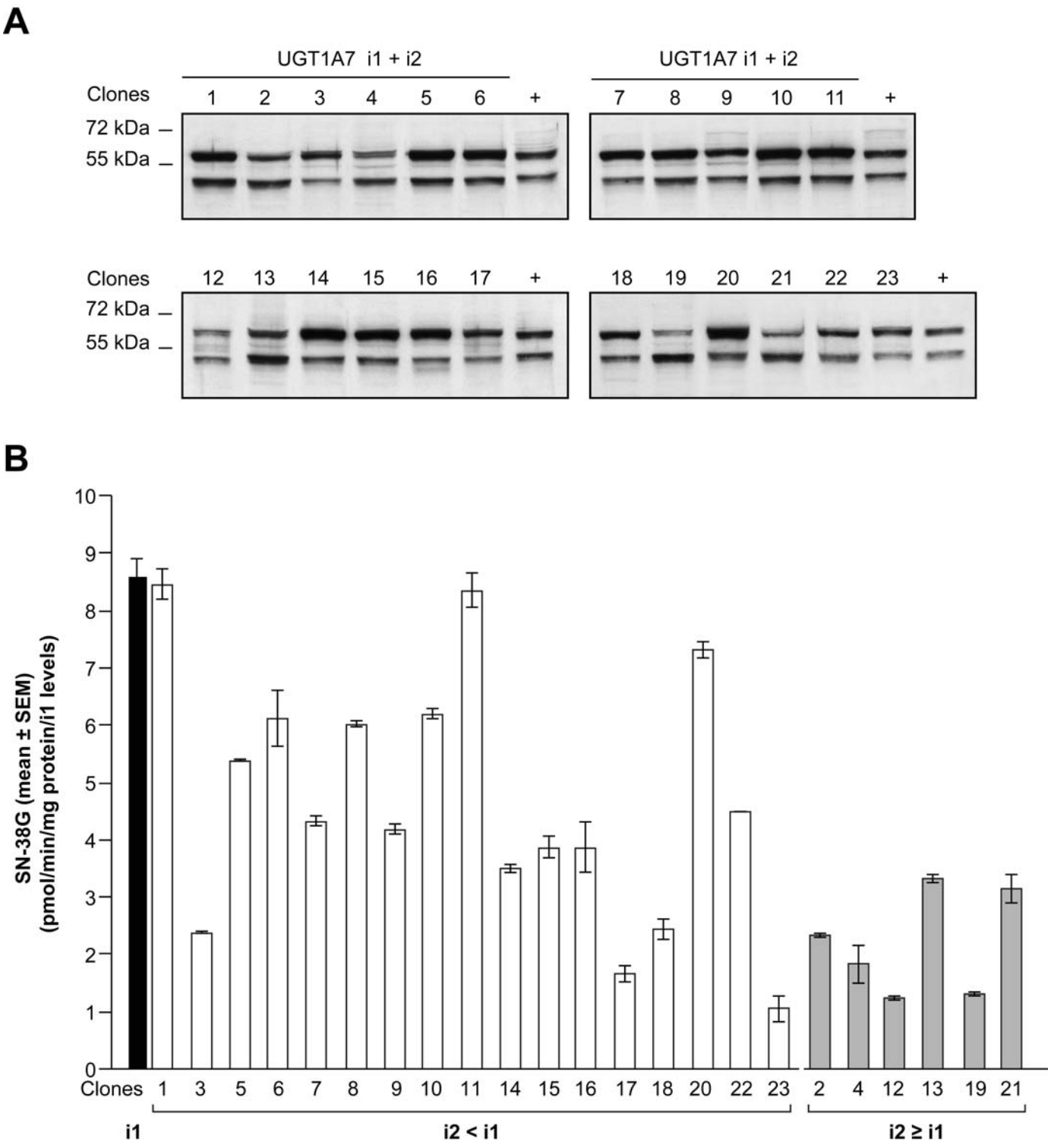
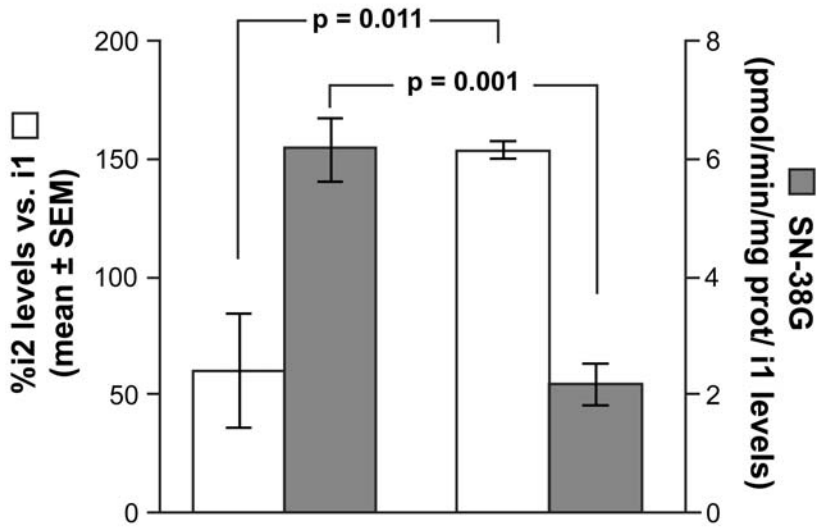

A
UGT1A7 i1 + i2
UGT1A7 i1 + i2
Clones
1 2 3 4 5 6 +
7 8 9 10 11 +
72 kDa
55 kDa
Clones
12 13 14 15 16 17 +
18 19 20 21 22 23 +
72 kDa
55 kDa
B
SN-38G (mean ± SEM)
(pmol/min/mg protein/i1 levels)
0 1 2 3 4 5 6 7 8 9 10
Clones 1 3 5 6 7 8 9 10 11 14 15 16 17 18 20 22 23 2 4 12 13 19 21
i1
i2 < i1
i2 ≥ i1

Figure 2

A



Clones (n)	i2 < i1 (17)	i2 ≥ i1 (6)
inhibition vs i1 (%) (mean ± SEM)	45.5 ± 6.2	74.5 ± 4.2

B