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Successful in vitro fertilization and generation of transgenics in Black and Tan Brachyury (BTBR) mice

Mieke Baan^a, Kathleen J. Krentz^b, Danielle A. Fontaine^a, and Dawn Belt Davis^{a,c}

^aDepartment of Medicine, Division of Endocrinology, Diabetes and Metabolism, University of Wisconsin-Madison, 5158 MFCB, 1685 Highland Ave, Madison, WI 53705

^bUniversity of Wisconsin-Madison Biotechnology Center, 425 Henry Mall, Madison, WI 53706

^cWilliam S. Middleton Memorial Veterans Hospital, 2500 Overlook Terrace, Madison, WI 53705

Abstract

The Black and Tan Brachyury (BTBR) mouse strain is a valuable model for the study of long-term complications from obesity-induced type 2 diabetes mellitus and autism spectrum disorder. Due to technical difficulties with assisted reproduction, genetically modified animals on this background have previously been generated through extensive backcrossing, which is expensive and time-consuming. We successfully generated two separate transgenic mouse lines after direct zygote microinjection into this background strain. Additionally, we developed in vitro fertilization (IVF) methods for the BTBR mouse. We found low rates of fertilization and implantation in this strain, and identified the BTBR oocyte as the primary culprit of low success with BTBR IVF. We achieved an increase in live born pups from 5.9% to 35.6% with IVF in the BTBR strain by use of BTBR females at a younger age (18–25 days), collection of oocytes 15–17 hours after superovulation, and the use of supplemented fertilization media. This method eliminates the need for time consuming assisted embryo manipulations that are otherwise required for success with BTBR oocytes. This advancement provides an exciting opportunity to directly generate BTBR transgenics and gene-edited mice using both traditional and emerging genomic editing techniques, such as CRISPR/Cas9. These methods also allow effective colony preservation and rederivation with these strains. To our knowledge, this is the first report describing embryo manipulations in BTBR mice.

Keywords

BTBR; in vitro fertilization; embryo transfer; transgenic mouse model; diabetes mellitus; autism

Corresponding author: Dawn Belt Davis, 4147 MFCB, 1685 Highland Ave, Madison, WI 53705, phone: 608-263-2443 fax: 608-263-9983, dbd@medicine.wisc.edu.

Ethical approval

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted.

Conflict of Interests

The authors declare that they have no conflict of interest.

Introduction

Animal models for human disease provide invaluable insight into pathophysiology and generate important clues for therapeutic discovery. *In vitro* reproductive techniques, embryonic stem cell technology, homologous DNA recombination, and Clustered Regularly-Interspaced Short Palindromic Repeats (CRISPR/Cas9) technology make it possible to alter the genome at targeted locations in mice. This has enabled the study of specific genes in disease development and progression. Mice are the most commonly used mammalian animal model because their genetic makeup is easy to manipulate, their reproductive cycle is short and prolific, and their husbandry is comparatively inexpensive.

Diabetes research makes use of many different mouse models (Dhuria et al. 2015; Heinonen et al. 2015). BTBR mice, the focus of this report, reliably develop obesity-associated diabetes mellitus (DM) after introduction of the spontaneous leptin-*ob* gene mutation (Clee et al. 2005; Hudkins et al. 2010). In mice homozygous for this mutation, the leptin hormone is unable to interact with its receptor, resulting in increased appetite, obesity, insulin resistance, and decreased glucose utilization (Anubhuti and Arora 2008). By 10 weeks of age, BTBR *ob/ob* mice are extremely obese and markedly hyperglycemic (Alpers and Hudkins 2011; Betz and Conway 2014; Clee et al. 2005; O'Brien et al. 2015; Stoeckl et al. 2000). The obese BTBR can serve as a model of type 2 diabetes (T2DM). The BTBR strain fails to adaptively stimulate proliferation of pancreatic islet cells and upregulate insulin production in response to obesity and insulin resistance (Keller et al. 2008). Therefore, BTBR mice have been used as a model to study pancreatic β -cell proliferation and survival (Chen et al. 2008; Davis et al. 2010). In addition, these mice reliably develop diabetic complications relevant to disease progression in humans, such as diabetic cardiomyopathy, nephropathy, and neuropathy (Alpers and Hudkins 2011; Hudkins et al. 2010; Lindstrom 2007; O'Brien et al. 2015). Because of these characteristics, the *ob/ob* BTBR mice are an excellent model to study the development of T2DM and its complications.

BTBR mice have also been widely used to study autism spectrum disorder (ASD). They naturally display behaviors that are consistent with the three diagnostic criteria for ASD in humans; impaired social interaction, communication deficits, and stereotyped repetitive behaviors (Meyza et al. 2013). BTBR mice consistently score low on tests that evaluate social interactions, they are deficient in ultrasonic vocalizations and urinary scent marking, and they display spontaneous stereotyped repetitive behaviors such as circling, jumping, excessive self-grooming, and digging (Careaga et al. 2015; McFarlane et al. 2008; McTighe et al. 2013; Meyza et al. 2013). In addition, structural brain development and neurogenesis in BTBR mice is abnormal, resulting in the complete absence of the *corpus callosum*, and a reduced number of neurosynapses within the hippocampus (Meyza et al. 2013). Because of the behavioral and physiological similarities with individuals diagnosed with ASD, BTBR mice are used extensively to study the mechanisms underlying the autistic phenotype, to discover biomarkers for the disease, and to develop treatment strategies.

In vitro fertilization (IVF) and embryo transfer (ET) are important steps in the generation and maintenance of genetically engineered animal models. To modify the genome, zygotes are produced either *in vivo* or in culture via IVF. The genetic makeup of these zygotes is

altered by microinjection of a transgenic DNA construct or the recently developed CRISPR/Cas9 components. After manipulation, the modified embryos are transferred into recipient pseudopregnant females and give rise to engineered offspring. Thus, production of adequate numbers of good quality mouse zygotes is of pivotal importance in the generation of gene-edited mouse lines. Additionally, to maintain or rederive transgenic lines, successful IVF and ET is needed.

Reproductive parameters such as IVF capacity have been examined for several other strains of mice, including the widely used C57BL/6J mouse (Bath 2010; Byers et al. 2006; Guan et al. 2014; Mochida et al. 2014; Ostermeier et al. 2008; Takeo et al. 2008; Takeo and Nakagata 2011; Vasudevan et al. 2010; Vasudevan and Szein 2011; Vergara et al. 1997). However, despite its unique suitability as a model for T2DM and autism, the BTBR mouse has been relatively underutilized for embryo manipulations, and to our knowledge, there are no published data on transgenic model development or IVF success rates in BTBR mice. In our initial work with this strain, we identified several challenges that limited assisted reproduction. To facilitate more widespread use of the BTBR strain in transgenic research, it is necessary to optimize assisted reproduction techniques.

The aim of this paper is to describe our successful methods for generating two transgenic BTBR lines and delineate the reproductive capability of BTBR mice using IVF.

Materials and Methods

All experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of the University of Wisconsin. Mouse strains BTBR, C57BL/6J, B6D2F1, FVB were initially obtained from The Jackson Laboratories (Bar Harbor, ME) and further bred and maintained on a 12:12-hour light-dark cycle (17:00–05:00 dark), at 22±2°C ambient temperature, and 30–70% humidity.

Superovulation procedures were performed as described previously (Behringer et al. 2004). Briefly, 21–27 day old (C57BL/6J) or 18–25 day old (BTBR) female mice were superovulated by intraperitoneal injection of 5 international units (IU) of Pregnant Mare Serum Gonadotropin (PMSG, National Hormone and Peptide Program (NHPP)), followed by 5 IU human Chorionic Gonadotropin (hCG, NHPP) 47–48 hours later. Hormones were administered at 13:00–16:00 hours for pronuclear microinjections and 18:00–20:00 hours for IVF procedures. All solutions used for culture experiments were pre-equilibrated at 37°C, 5% CO₂ and covered with light white mineral oil.

Transgenic mouse lines containing the Mouse Insulin Promoter (MIP) driving murine Forkhead box M1 (FoxM1) expression (MIP-FoxM1) or the Rat Insulin Promoter (RIP) driving murine Survivin (SVV) expression (RIP-SVV) were developed. The MIP-FoxM1 gene construct has been described previously (Baan et al. 2015). The RIP-SVV gene construct was obtained from Dario Altieri (Dohi et al. 2006). For generation of transgenic mice, one-cell fertilized embryos were produced for pronuclear microinjection by mating superovulated females to stud males (1:1). The following morning, females were

anesthetized with CO₂ and sacrificed, and the oviducts were excised. Cumulus oocyte complexes (COCs) were collected in M2 media, treated with hyaluronidase (0.3–0.5 mg/mL) to remove cumulus cells, and then allowed to mature in human tubular fluid (HTF) medium for at least 1 hour at 37°C and 5% CO₂ (Quinn et al. 1985). Embryos with two pronuclei were microinjected with purified transgene DNA at 5 ng/μL and then surgically transferred to the oviduct of pseudopregnant B6D2F1 females, as previously described (Behringer et al. 2004). Resulting offspring were genotyped at weaning. Polymerase chain reaction (PCR) was used for genotyping, with the following primers: for MIP-FoxM1 mice: F-CTTCTAGACATCAGTTTCCCTGGC and R-TGGCCAAATACTGGGCTTAC, yielding a 328-bp PCR product (Figure 1A). For RIP-SVV mice: F-TTCCTTCTACCTCTGAGGGTG and R-TCGAGGTCTGACTCAGCATTAG, yielding a 1100-bp PCR product (Figure 1B). Founders were bred to wildtype BTBR females. Transgene expression was confirmed in pancreatic islets isolated from 10–12 week old transgenic offspring ((Baan et al. 2015) and Figure 1C).

For initial IVF experiments (Tables 2 and 3), sperm from the vas deferens and cauda epididymides of proven breeder adult C57BL/6J or BTBR sires was placed into 1ml HTF medium and allowed to swim out for 10 minutes. For IVF using fresh sperm, five microliters (μL) of sperm was added to fertilization drops of 250 μL HTF. For cryopreservation experiments, sperm was collected in 18% raffinose/3% skim milk then frozen in the vapor phase of liquid nitrogen in cryovials (Behringer et al. 2004). For thawing, cryovials were placed in 37°C waterbath for 2 minutes then 10 μL of sperm was placed into fertilization drops of 250 μL HTF. For initial experiments (Table 2 and 3), superovulated females were sacrificed 12–13 hours after hCG administration, immediately after sperm placement into fertilization drops. The oviducts were excised, COCs collected, and placed into fertilization drops containing the sperm. Where indicated, we performed assisted in vitro fertilization (AIVF). For AIVF, cumulus cells were manually removed from the oocytes using hyaluronidase, and the zona pellucida was thinned using acid tyrode's. After 4–5 hours of co-incubation in the fertilization drops, detached cumulus cells and excess sperm was removed by washing oocytes three times in 200 μL drops of HTF. Oocytes were cultured overnight in 37°C and 5%.

With advancements in the field and our own experience with the BTBR IVF results, the following modifications were made (for data presented in Table 4); Sperm was taken from the cauda epididymidis only and collected in HTF that contained 1 mg/mL polyvinyl alcohol instead of bovine serum albumin (BSA) and was supplemented with 0.75 mM methyl-β-cyclodextrin (MBCD) (Takeo and Nakagata 2010). Harvesting of COCs was now performed 15–17 hours after hCG administration. COCs were then cultured for 1h in high performance HTF (hpHTF- containing 4% BSA, 1mM glutathione (GSH) and additional CaCl (0.755 gr/L total concentration)) prior to fertilization with sperm.

Data presented in this report reflect fertilization rates obtained in HTF medium (Quinn et al. 1985) or hpHTF medium as indicated {Takeo, 2011 #563}. In vitro fertilization rates were evaluated the morning after fertilization, and were calculated by dividing the number of two cell embryos by the total number of oocytes collected, multiplied by 100%.

Results

The BTBR strain is described as exceptional breeders on The Jackson Laboratory's website. BTBR females are good mothers and tend to produce large litters (greater than 12 pups/litter) in our colony. Young BTBR females respond well to superovulation with exogenous hormones, usually producing greater than 40 oocytes per female. BTBR males have high sperm counts when evaluated on a computer-assisted sperm analyzer (31% motile, 61.4 million sperm/mL). However, in our initial experiments *in vitro* fertility rates were low in comparison to other strains.

We developed two transgenic mouse lines in the BTBR background. After superovulation and natural fertilization, BTBR zygotes with two pronuclei were microinjected with linearized transgene DNA, and 6–44 zygotes were transplanted into a pseudopregnant recipient female (Table 1). Because the number of fertilized embryos with visible pronuclei was low in early attempts at microinjection, we co-transferred non-injected FVB embryos (control embryos) with the microinjected BTBR embryos to improve implantation and live birth rates. These control mice could easily be distinguished at birth and weaning due to albino coat color. Of the transplanted, microinjected BTBR embryos we saw a low rate of live births, with only 7.6 and 14.8 % of implanted embryos carried to term (Table 1). Presence of the transgene in the genomic DNA was confirmed with PCR of DNA extracted from tail or ear snips (Figure 1). One founder MIP-FoxM1 male (#355) was obtained out of 11 live pups (9%), and 3 founder males came from 16 live pups in the MIP-SVV line (20%, Table 1). Transgene expression under control of the insulin promoter in the pancreatic beta-cell specifically was confirmed by quantitative PCR in isolated pancreatic islets from MIP-FoxM1 (previously described in Baan, et al., 2015) and RIP-SVV mice. The three RIP-SVV founder lines had upregulation of survivin mRNA in the pancreatic islet of 28-, 67-, and 64-fold (Figure 1C). Overall, while ultimately successful, we found challenges in the generation of BTBR transgenic mice related to low embryo production and low post-injection survival rates.

Successful generation and maintenance of knockout and transgenic mouse lines may be dependent on the ability to perform IVF. We set out to characterize the ability to perform IVF in the BTBR strain. As mentioned above, the BTBR strain responds well to superovulation. Notably, we achieved these high rates of superovulation by using 18–25 day old BTBR females, younger than the typical age in other strains. Success with superovulation was diminished in 26–35 day old BTBR females (data not shown). The IVF fertilization rate with fresh sperm in BTBR mice was around 18% in our facility (Table 2). In comparison, fertilization rate in C57BL/6J mice with fresh sperm was markedly higher (43.8%, Table 2). The lower fertilization rates in BTBR mice were accompanied by lower percentages of live born pups, i.e. 5.9% in BTBR vs 52.2% in C57BL/6J. Manual removal of the cumulus cells and thinning of the zona pellucida, *i.e.* the assisted-IVF procedure (AIVF), increased the percentage of live born pups from 5.9 to 25.7% in the BTBR mice (Table 2). Cryogenic preservation of sperm is a common method for maintenance of transgenic mouse lines, so we wanted to test whether frozen BTBR sperm were effective for IVF. Fertilization rates were reduced using frozen sperm to only 10.0% and the percentage of live born pups from transplanted embryos was 16.3%. Therefore, frozen BTBR sperm are adequate for

transgenic line maintenance, but higher numbers of oocytes should be used to ensure success.

Hybrid studies were performed to investigate the cause of the poor fertilization rates of the BTBR strain. Cross fertilizations between BTBR and C57BL/6J gametes showed that IVF fertilization rates in BTBR mice are limited by oocyte factors (Table 3). Without the use of AIVF, fertilization rates of BTBR oocytes were very low (0 and 4.9%), regardless of whether C57BL/6J or BTBR sperm were used. Conversely, BTBR sperm successfully fertilized 79.2% of C57BL/6J oocytes (Table 3), resulting in 60% live pups after embryo transfer (data not shown). Notably, the use of AIVF markedly improved fertilization rates of BTBR oocytes, indicating that with BTBR oocytes AIVF is necessary for adequate fertilization to occur. These data clearly show that BTBR cumulus oocyte complex factors play an important role in low fertilization success in this strain.

Data summarized in Tables 1, 2 and 3 were obtained prior to the introduction of high performance HTF (hpHTF), and show that transgenesis, IVF and ET in the BTBR mice are possible, even with the use of frozen sperm, albeit with modest success. More recently, modification of HTF medium (hpHTF) by supplementation of additional calcium and fresh glutathione (GSH) greatly improved assisted reproductive success (Takeo and Nakagata 2010; Takeo and Nakagata 2011). In our facility, fertilization rates in a variety of mouse strains also increased after introduction of hpHTF (data not shown). In addition, fertilization success rates further improved when the time interval between administration of hCG and harvest of oocytes was extended from 12–13h to 15–17h. Extending this time interval in combination with the use of hpHTF and other minor modifications (see Materials and Methods) resulted in higher fertilization rates in both wildtype C57BL/6J and wildtype BTBR mice (Table 4). BTBR fertilization rates were now 41.3% without time-consuming AIVF (compared to 18.2% in Table 2) and live birth rates were now 35.6% (compared to 5.9% in Table 2). Importantly, with these new methods BTBR fertilization and live birth rates still remain below those of C57BL/6J but are now high enough to reliably allow successful IVF in this strain.

Discussion

This is the first report of embryo manipulation in the BTBR mouse strain. Previous studies have compared fertilization success between different strains of mice, but none have included the BTBR strain (Byers et al. 2006; Mochida et al. 2014; Szein et al. 2000; Vasudevan et al. 2010). Although good spontaneous breeders, reproductive success is low in BTBR mice after IVF, manipulation of the genome, and embryo transfer. This has impeded the use of BTBR as an animal model for human disease. Here, we show that it is possible to directly genetically engineer a BTBR embryo, eliminating the need for extensive backcrossing from other background strains.

BTBR males produce high quality sperm, and our crossbreeding studies show that BTBR oocyte factors are responsible for the low in vitro fertilization rates. Fertility rates are impacted by the strain of mouse, superovulation protocols, and methods of oocyte maturation and sperm capacitation (Hillier et al. 1985; Hino et al. 2011; Kanter et al. 2004;

Vasudevan and Sztein 2012). 129S/T mice are another strain with low reported in vitro fertility rates (Hino et al. 2011). In these mice, the oocyte's zona pellucida had an increased resistance to sperm penetration when they were harvested 17h rather than 14h after superovulation (Hino et al. 2011). In the present study, BTBR fertilization rates improved with oocyte collection at 15–17 hours after hGH administration, rather than at 12–13h, confirming that protocol optimization is needed for each individual mouse strain. Furthermore, when using standard HTF, the percentage of live born pups was markedly improved with assisted IVF, i.e. the manual removal of the cumulus cells and thinning of zona pellucida. Our cross-fertilization studies show that oocyte factors are limiting fertilization success in BTBR mice, although the mechanism is not clear.

Despite these obstacles and prior to the introduction of hpHTF, we successfully generated two transgenic mouse lines in a BTBR background, i.e. the MIP-FoxM1 and the RIP-SVV mouse. Notably, in our facility, superovulation is more successful when 18–25 day old BTBR females are used, rather than at 26–35 days. Juvenile BTBR mice gain weight more rapidly in comparison with other strains, and increased body weight contributes to reduced superovulation rates. In addition, we co-transferred unmanipulated embryos from a different mouse strain to improve the percentage of live pups from microinjected BTBR embryos. We hypothesize that transfer of these non-injected embryos increased overall litter size, increased the ability of the mother to maintain the pregnancy and prevented early embryonic or early neonatal death. Embryo-related factors might also have played a role, as the mere presence of a transgene can affect embryo survival (Vasudevan et al. 2010).

With the introduction of hpHTF, we ultimately achieved a fertilization rate of 41.3% in BTBR mice, and 35.6% live born pups. hpHTF media is supplemented with glutathione, which aids in oocyte manipulation by mimicking hyaluronidase and acid tyrodes without the laborious technique required to use those chemicals. Additionally, MBCD enhances sperm capacitation and motility. With this advancement, the use of standard HTF, even in combination with AIVF, has become obsolete. However, the information obtained with these older methods is still informative in terms of identification of the challenges with the BTBR strain. Some facilities may still be using standard HTF media, and therefore the use of AIVF will be necessary for success with BTBR IVF.

This is the first report to describe a protocol for the development of genetically-engineered BTBR mice. BTBR mice are uniquely suitable for the study of type-2 diabetes mellitus and its complications and for the study of autism spectrum disorder. Our work provides the reader with a guide to achieve better reproductive success with this strain. Production of adequate numbers of good-quality BTBR embryos offers an exciting opportunity to generate genetically edited BTBR mice with the new CRISPR/Cas9 technique in the future.

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Figure 1. MIP-FoxM1 and RIP-SVV BTBR transgenic line development

Gel electrophoresis of PCR products from genomic DNA of MIP-FoxM1 (#355, A) and RIP-SVV (# 500 and 508, B) founders and non-transgenic littermates, demonstrating presence of the transgene in founder mice. C) Expression of survivin mRNA in isolated pancreatic islets of all three RIP-SVV founder lines compared to wild type BTBR littermates.

Table 1

Generation of transgenic BTBR founders

MIP-FoxM1= mouse insulin promoter (MIP) – murine FoxM1 transgene construct, RIP-SVV = rat insulin promoter (RIP) – murine Survivin transgene construct. MI = microinjected, # = number, %ET = percentage of embryos transferred, %BP= percentage of live BTBR pups

Transgene	MI BTBR embryos #	Control embryos #	Recipient females # (% pregnant)	BTBR pups # (% ET)	Transgenic pups # (%BP)	Control pups # (%ET)
MIP-FoxM1	158	8	5 (100)	12 (7.6)	1 (9.0)	8 (100)
RIP-SVV	108	10	5 (100)	16 (14.8)	3 (20.0)	7 (70)

Table 2

IVF success rates for BTBR and C57BL/6J mice using fresh vs. frozen sperm and standard vs. assisted IVF

Results were obtained prior to the introduction of high performance HTF medium. AIVF = assisted *in vitro* fertilization, # = number, F= female, O=oocytes, 2CE = 2 cell-stage embryo, FR= fertilization rate (development of 2CE), R = recipient mouse, ET = embryos transferred into pregnant recipients, live born = (# live pups/ET)x100%.

Gamete strain (# F)	Sperm type	AIVF	oocytes #(#O/F)	2CE # (FR%)	recipients # (#2CE/R)	pregnant # (%)	ET	pups	live born %
BTBR (14)	fresh	No	1408 (100.6)	256 (18.2)	11 (17)	1 (9)	17	1	5.9
BTBR (3)	fresh	Yes	306 (102)	59 (19.3)	3 (11.7)	3 (100)	35	9	25.7
BTBR (7)	frozen	Yes	478 (68.3)	48 (10.0)	3 (15)	3 (100)	43	7	16.3
C57BL/6 J (124)	fresh	No	6356 (51.3)	2782 (43.8)	32 (108)	27 (84.4)	347	181	52.2
C57BL/6 J (62)	frozen	Yes	5052 (81.5)	1672 (33.1)	52 (37)	45 (86.6)	661	240	36.3

Table 3
In vitro cross-fertilization of BTBR and C57BL/6J gametes

Results were obtained prior to the introduction of high performance HTF medium. # = number, O = oocytes, F = females, AIVF = assisted *in vitro* fertilization, 2CE = 2 cell-stage embryo, FR= fertilization rate (development of 2CE).

Oocyte - strain (# F)	Sperm - strain (fresh)	AIVF	oocytes # (#O/F)	2CE # (FR%)
C57BL/6J(2)	C57BL6/J	No	97 (48.5)	36 (37.1)
C57BL/6J(2)	BTBR	No	101 (50.5)	80 (79.2)
BTBR (3)	C57BL/6J	No	88 (29.3)	0 (0)
BTBR (4.5)	C57BL/6J	Yes	143 (31.8)	35 (24.6)
BTBR (3)	BTBR	No	106 (35.3)	5 (4.9)
BTBR (4.5)	BTBR	Yes	145 (32.2)	41 (29.1)

Table 4
In vitro fertilization results using high performance HTF medium

= number, F = female, O = oocytes, 2CE = 2 cell-stage embryo, FR = fertilization rate (development of 2CE), R = recipient mouse, ET = embryos transferred, live born = (#pups/IE)x100%

Donor strain (# F)	Sperm	oocytes # (#O/F)	2CE # (FR%)	recipients # (2CE/R)	Pregnant # (%)	#pups/#ET	live born %
C57BL/6J (68)	fresh	1359 (20.0)	1136 (83.6)	8 (17.75)	8 (100)	68/142	47.9
BTBR (19)	fresh	828 (43.6)	342 (41.3)	3 (19.7)	3 (100)	21/59	35.6