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Predominant contribution of L-type amino acid transporter to 4-borono-2-^{18}F-fluoro-phenylalanine uptake in human glioblastoma cells☆

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ABSTRACT

Introduction: 4-Borono-2-^{18}F-fluoro-phenylalanine (^{18}F-FBPA) has been used to anticipate the therapeutic effects of boron neutron capture therapy (BNCT) with 4-borono-L-phenylalanine (BPA). Similarly, L-[methyl-^{11}C]-methionine (^{11}C-MET), the most popular amino acid PET tracer, is a possible candidate for this purpose. We investigated the transport mechanism of ^{18}F-FBPA and compared it with that of ^{14}C-MET in human glioblastoma cell lines.

Methods: Uptake of ^{18}F-FBPA and ^{14}C-MET was examined in A172, T98G, and U-87MG cells using 2-aminobicyclo-(2.2.1)-heptane-2-carboxylic acid (a system L-specific substrate), 2-(methylamino)-isobutyric acid (a system A-specific substrate), and BPA. Gene expression was analyzed by quantitative real time polymerase chain reaction.

Results: System L was mainly involved in the uptake of ^{18}F-FBPA (74.5%–81.1% of total uptake) and ^{14}C-MET (48.3%–59.4%). System A and ASC also contributed to the uptake of ^{14}C-MET. Inhibition experiments revealed that BPA significantly decreased the uptake of ^{18}F-FBPA, whereas 31%–42% of total ^{14}C-MET uptake was transported by BPA non-sensitive transporters. In addition, ^{18}F-FBPA uptake correlated with LAT1 and total LAT expressions.

Conclusion: This study demonstrated that ^{18}F-FBPA was predominantly transported by system L in human glioblastoma cells compared to ^{14}C-MET. Although further studies are needed to elucidate the correlation between ^{18}F-FBPA uptake and BPA content in tumor tissues, ^{18}F-FBPA is suitable for the selection of patients who benefit from BNCT with BPA.

1. Introduction

Boron neutron capture therapy (BNCT) is radiotherapy using high linear energy transfer (LET) alpha particle (^{4}He) and recoiling lithium (^{7}Li), which are produced by a nuclear fission reaction of ^{10}B with low energy thermal neutrons. Clinical interest in BNCT has focused primarily on patients with high grade glioma [1–3]. The success of BNCT requires sufficient accumulation of ^{10}B in cancer tissues. The representative ^{10}B carrier used in clinical trials is 4-borono-L-phenylalanine (BPA), and a preferable tumor to normal tissue ratio is greater than 3–5 [4]. Therefore, estimation of ^{10}B content in cancer tissue helps to anticipate the therapeutic potential of BNCT.

An ^{18}F-labelled analog of BPA, 4-borono-2-^{18}F-fluoro-phenylalanine (^{18}F-FBPA) has been developed to predict ^{10}B concentration in tumors [5,6]. Imahori et al. indicated that the ^{10}B concentration and kinetics of BPA can be estimated using the rate constants of ^{18}F-FBPA-positron emission tomography (PET) [7,8]. ^{18}F-FBPA and BPA are thought to be transported via system L, which recognizes neutral amino acids with large, branched, or aromatic side chains like phenylalanine as substrates. Thus, four isoforms of system L, LAT1, LAT2, LAT3, and LAT4, have been identified so far [9–12]. In particular, overexpression of LAT1 is widely found in many tumors [13,14]. Detta et al. found that the uptake of BPA in human brain tumor samples was inhibited by phenylalanine and 2-aminobicyclo-(2.2.1)-heptane-2-carboxylic acid (BCH), indicating the significant involvement of system L [15]. However, the contribution of other transporters, such as system A and ASC, in the uptake of BPA was not investigated. Thus, the transporter systems involved in the uptake of ^{18}F-FBPA in glioma cells still remain to be determined.

^{18}F-FBPA is synthesized only in certain hospitals, thus, its utility seems clinically challenging. However, L-[methyl-^{11}C]-methionine (^{11}C-MET), which is the most popular amino acid PET tracer, available in many hospitals, is also a candidate for screening of patients

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applicable to BNCT with BPA. PET with ^{11}C-MET is useful for diagnosis of brain tumors [16,17]. Moreover, ^{11}C-MET is also transported into cells by system L [18,19]. To determine the difference between the two PET tracers, we investigated the transport mechanism of ^{18}F-FBPA and ^{14}C-MET using three human glioblastoma cell lines. Direct comparison of both tracers gives us valuable information to estimate the therapeutic benefits of BNCT with BPA.

2. Materials and methods

2.1. Cell culture

Three human glioblastoma cell lines used were A172, T98G, (DS Pharma Biomedical Co., Ltd., Osaka, Japan) and U-87 MG (American Type Culture Collection, Manassas, VA, USA). A172 cells were cultured in Dulbecco's modified Eagle's medium (4.5 g/L glucose; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS). T98G and U-87MG were cultured in Eagle's minimum essential medium (Invitrogen) supplemented with 10% FBS. All cell lines were maintained in a humidified atmosphere of 5% CO_2 in air at 37 °C.

2.2. Synthesis of ^{18}F-FBPA

^{18}F-FBPA was synthesized by direct electrophilic radiofluorination of BPA (Sigma-Aldrich, St. Louis, MO, USA) using ^{18}F-acetyl hypofluorite as described previously [20]. Purification of ^{18}F-FBPA was performed by HPLC using YMC-Pack ODS-A column (20 × 150 mm; YMC, Kyoto, Japan) eluted with 0.1% acetic acid at a flow rate of 10 ml/min. The radiochemical purity was determined by HPLC. The radiochemical purity and the specific activity of ^{18}F-FBPA were >99.5% and 25 MBq/μmol, respectively.

2.3. In vitro uptake of ^{18}F-FBPA and ^{14}C-MET in human glioblastoma cell lines

In vitro uptake studies were carried out as described previously [21]. L-[methyl-^{14}C] methionine (^{14}C-MET) was purchased from American Radiolabeled Chemicals Co. (St. Louis, MO, USA). Tumor cells (1×10^5 cells/well) were seeded in 24-well plates. The uptake studies were performed on the subsequent day after seeding. The sodium-containing assay buffer was composed of phosphate-buffer saline (Na^+-PBS) supplemented with 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 5.6 mM D-glucose, 0.9 mM $CaCl_2$, and 0.5 mM $MgCl_2$. In the sodium-free assay buffer (Na^+-free PBS), NaCl and Na_2HPO_4 were replaced by choline chloride and K_2HPO_4, respectively. Preliminary experiments to determine the time curse of ^{18}F-FBPA uptake into T98G cells indicated that the uptake was linearly dependent on incubation time up to 30 min (data not shown). Thus, the uptake of ^{18}F-FBPA and ^{14}C-MET was measured for 30 min. After removing culture medium, the cells were pre-incubated with 500 μl of the assay buffer for 10 min at 37 °C. The cells were then incubated with 500 μl of the assay buffer containing ^{18}F-FBPA (185 kBq) or ^{14}C-MET (18.5 kBq) for 30 min at 37 °C. After incubation, the cells were washed twice with ice-cold assay buffer and dissolved in 0.1 N NaOH. The radioactivity in the cells was measured with a gamma counter (AccuFLEX γ7001; Aloka, Tokyo, Japan) and a liquid scintillation counter (Tri-Carb 3110TR; PerkinElmer, Waltham, MA, USA).

To characterize the transport system, we performed the inhibition experiment with inhibitors: 2-aminobicyclo-(2.2.1)-heptane-2-carboxylic acid (BCH; Sigma-Aldrich) for system L, 2-(methylamino)-isobutyric acid (MeAIB; Sigma-Aldrich) for system A, and BPA at concentrations indicated in figures. To calculate the contribution of amino acid transporter systems, we used the methods reported by Kobayashi et al. [18]. In brief, uptake of ^{18}F-FBPA and ^{14}C-MET in the absence of inhibitors was used as the control (100%). System A and ASC transport was calculated by subtracting the tracer uptake in the presence of MeAIB from uptake in the Na^+-PBS control and Na^+-free PBS control, respectively. System L transport was calculated by subtracting tracer uptake in the presence of BCH from the uptake in the Na^+-free PBS control. Finally, System PAT transport was calculated by subtracting the tracer uptake in the presence of MeAIB from the Na^+-free PBS control.

2.4. qRT-PCR analysis

Total RNA was extracted from A172, T98G, and U-87MG cells using an RNeasy Mini kit (QIAGEN, Tokyo, Japan). cDNA was synthesized from total RNA using an AffinityScript QPCR cDNA Synthesis kit (Agilent Technologies, Santa Clara, CA, USA). The thermal profile of the reverse transcription was as follows: 5 min at 25 °C, 15 min at 42 °C for and 5 min at 95 °C. System A (SNAT1, SNAT2, SNAT4) and ASC (ASCT1, ASCT2) as Na^+-dependent transporters, system L (LAT1, LAT2, LAT3, LAT4) as Na^+-independent transporter, and 4F2hc which forms heterodimers with LAT1 and LAT2 were analyzed. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin (ACTB) were used as internal controls. Primers for each target gene were synthesized by Nihon Gene Research Laboratories (Table 1; Miyagi, Japan). Quantitative real time polymerase chain reaction (qRT-PCR) was performed on an Mx3005P (Agilent Technologies) using Brilliant III Fast SYBR Green QPCR Master Mix (Agilent Technologies). The thermal profile of QPCR reaction was as follows: 3 min at 95 °C for 1 cycle, 5 s at 95 °C followed by 20 s at 60 °C for 40 cycles, 1 min at 95 °C followed by 30 s at 55 °C and 30 s at 95 °C for 1 cycle. The mRNA copy number was calculated from standard curves generated by amplifying serial dilutions of a known concentration of purified amplicons. Expression data were normalized against the average copy number of housekeeping genes. All samples were analyzed in triplicate.

Table 1
Sequences of primers.

Official Symbol	Alias	Primer	Sequence
SLC38A1	SNAT1	Forward	attttgggactcgcctttg
		Reverse	agcaatgtcactgaagtcaaaagt
SLC38A2	SNAT2	Forward	cctatgaaatctgtacaaaagattgg
		Reverse	ttgtgtacccaatccaaaacaa
SLC38A4	SNAT4	Forward	tgttctggtcatccttgtgc
		Reverse	aaaactgctggaagaataaaaatcag
SLC1A4	ASCT1	Forward	tttgcgacagcatttgctac
		Reverse	gcacttcatcatagagggaagg
SLC1A5	ASCT2	Forward	gaggaatatcaccggaacca
		Reverse	aggatgttcatcccctcca
SLC7A7	y^+LAT1	Forward	cctgcttatatccaggaccaa
		Reverse	ggccacttcatactcagtgct
SLC7A6	y^+LAT2	Forward	cctatccctgctttactgttcaa
		Reverse	aagctgaagtagttgataagctgga
SLC7A5	LAT1	Forward	gtggaaaaacaagcccaagt
		Reverse	gcatgagcttctgacacagg
SLC7A8	LAT2	Forward	ttgccaatgtcgcttatgtc
		Reverse	ggagcttctctccaaaagtcac
SLC43A1	LAT3	Forward	tttggtggcatctgcctaa
		Reverse	attaacgtggagcgcaggt
SLC43A2	LAT4	Forward	cagggagaccctctgtgg
		Reverse	cggtagcagatcaggtagagc
SLC3A2	4F2hc	Forward	taccggggtgagaactcgt
		Reverse	cagccaaaactccagagcat
ACTB	actin, beta	Forward	ccaaccgcgagaagatga
		Reverse	ccagaggcgtacagggatag
GAPDH	-	Forward	agccacatcgctcagacac
		Reverse	gcccaatacgaccaaatcc

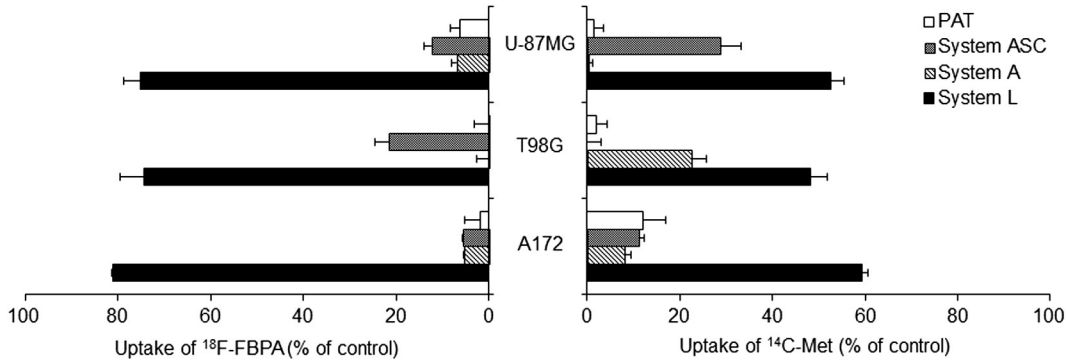

Fig. 1. Contribution of amino acid transporters to ^{18} F-FBPA (left) and ^{14}C-MET (right) uptake in human glioblastoma cell lines. ^{18} F-FBPA or ^{14}C-MET was incubated with cells for 30 min (n = 4).

2.5. Statistical analysis

The correlations between tracer uptake and gene expression were analyzed using GraphPad Prism v5.04 (Graph Pad Software Inc., San Diego, CA, USA).

3. Results

3.1. Competitive inhibition study of ^{18} F-FBPA and ^{14}C-MET

We estimated the contribution of each amino acid transport system to ^{18} F-FBPA and ^{14}C-MET in A172, T98G, and U-87MG cells (Fig. 1). System L was predominantly involved in tumor uptake of ^{18} F-FBPA (74.5%–81.1% of total uptake). Contribution of system ASC and other transport systems in the uptake of ^{18} F-FBPA was 5.6%–21.6% and 1.9%–6.9%. Uptake of ^{14}C-MET was also mainly mediated by system L (48.3%–59.4%). In addition, 28.8% of total ^{14}C-MET uptake in U-87MG cells was mediated by system ASC. In T98G cells, system A contributed to 22.7% of total ^{14}C-MET uptake. Contribution of PAT, system ASC, and system A was 8.1%–12.1% in A172 cells.

3.2. Dose-dependent inhibition of FBPA and MET uptake by BPA

Uptake of ^{18} F-FBPA was dose-dependently inhibited by BPA in all cell lines (Fig. 2). BPA (1 mM) reduced the uptake of ^{18} F-FBPA to 2.1%–7.1% of control. On the other hand, the uptake of ^{14}C-MET was decreased by 1 mM BPA to 31.2% in A172, 42.2% in T98G and 32.4% in U-87MG.

3.3. Gene expression of amino acid transporters in A172, T98G and U-87MG

The gene expression of amino acid transporters was summarized in Table 2. The Na^+-dependent transporter systems, system A, and system ASC, were predominantly expressed in T98G cells. The expression of system ASC in U-87MG cells was higher than that in A172 cells. Among system L, LAT1 was highly expressed in T98G cells, while LAT4 was expressed in A172 cells.

3.4. Relationship between tracer uptakes and gene expressions

Fig. 3 showed that the uptake of ^{18} F-FBPA correlated with LAT1 and total LAT expression (r = 0.8576 for LAT1 and 0.9418 for total LAT). The uptake of ^{14}C-MET correlated with total LAT expression (r = 0.6691), but not with LAT1 expression. However, these correlations are statistically not significant.

4. Discussion

We investigated and compared the transport mechanism of ^{18} F-FBPA and ^{14}C-MET in human glioblastoma cell lines, A172, T98G, and U-87MG. The present study revealed that although system L is a main contributor for the uptake of ^{18} F-FBPA and ^{14}C-MET, other transporters also mediate the uptake of ^{14}C-MET.

BCH drastically inhibited the uptake of ^{18} F-FBPA in all glioblastoma cell lines, suggesting the major involvement of system L, containing LAT1, LAT2, LAT3, and LAT4. The uptake of BPA and ^{125}I-2-iodo-L-phenylalanine was inhibited by BCH and phenylalanine,

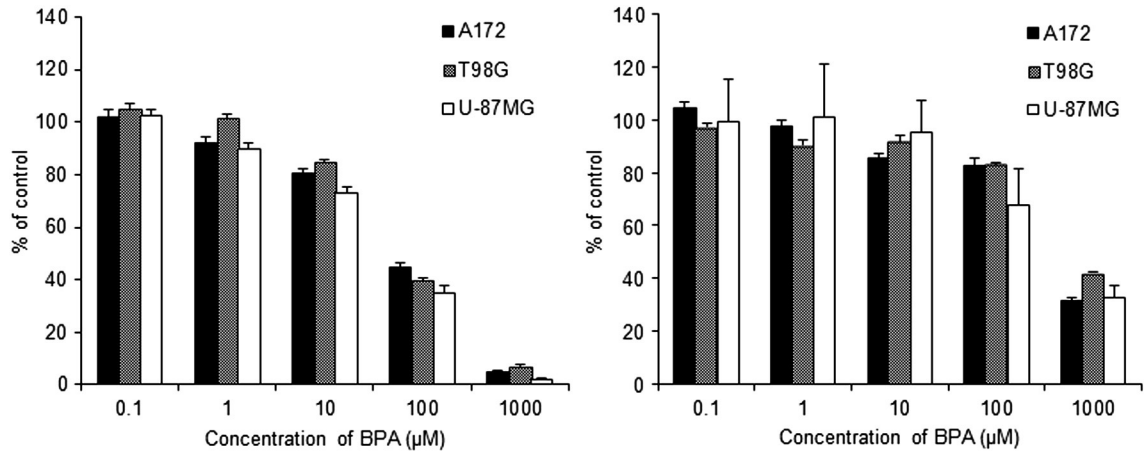

Fig. 2. Effect of BPA to ^{18} F-FBPA (left) and ^{14}C-MET (right) uptake in human glioblastoma cell lines. ^{18} F-FBPA or ^{14}C-MET was incubated with the indicated concentration of BPA and the cells for 30 min (n = 4).

Table 2
Expression of amino acid transporters in human glioblastoma cell lines.

	System	Family	A172	T98G	U-87MG
Na^+-dependent	A	SNAT1	14.14	21.50	24.23
		SNAT2	39.31	105.84	41.79
		SNAT4	0.18	ND	ND
	ASC	ASCT1	4.60	10.70	12.09
		ASCT2	10.10	88.86	41.84
	y^+L	yLAT1	ND	0.88	ND
		yLAT2	8.75	10.89	11.94
Na^+-independent	L	LAT1	15.67	59.01	16.19
		LAT2	1.67	0.64	2.44
		LAT3	1.66	1.66	0.49
		LAT4	42.59	9.19	3.17
Coupling factor	4F2hc	4F2hc	37.21	191.79	101.10

Data are expressed as copy number per 1000 copies of housekeeping genes.
ND: not determined.

supporting our results [15,22,23]. However, these studies have focused only on LAT1 because its expression is elevated in a variety of tumors, and LAT4 has not yet been well-characterized in tumor [14]. The qRT-PCR analysis revealed that ^{18}F-FBPA uptake demonstrated an enhanced correlation with total LAT, mainly LAT1 and LAT4, than LAT1 alone. Haase et al. reported higher expression of LAT4 than LAT1 in HT-29 colon cancer cells and FaDu head and neck cancer cells [24]. Phenylalanine is a substrate for LAT4 as well as LAT1 [10]. These data imply that LAT4 expression could be an important factor for ^{18}F-FBPA uptake in tumors.

Similarly, system L was predominantly involved in the uptake of ^{14}C-MET. Unlike ^{18}F-FBPA; however, other components, such as system ASC and system A, also contributed to the uptake of ^{14}C-MET. This resulted in a weak correlation between ^{14}C-MET uptake and total LAT expression, even though one of the reasons may be because of the small number of cell lines used in this study. Soriano-Garcia et al. indicated the involvement of four systems containing system L in MET transport [25]. Shotwell et al. reported that the uptake of MET in Chinese hamster ovary (CHO) cells was mediated by system L (51%), system ASC (22%), and system A (9%) [26]. Thus, uptake of ^{14}C-MET is regulated by a diverse expression of amino acid transporters.

Other reason for this weak correlation might be because the incorporation of ^{14}C-MET into protein contributes to the uptake of ^{14}C-MET. ^{14}C-MET is metabolized and then incorporated to protein. The reflux and the metabolism of ^{14}C-MET through the incubation for 30 min would not be negligible. Therefore, the uptake of ^{14}C-MET might not solely reflect the transport activity of ^{14}C-MET.

Our inhibition study and other previous studies have indicated that ^{14}C-MET was partly transported by BPA-non-sensitive transporters. Therefore, ^{11}C-MET-PET may overestimate the concentration of BPA in tumor tissues because of its compatibility with a broad range of amino acid transporters. In this study, SNAT2, ASCT2, and system L were expressed at various levels in glioblastoma cell lines. The expression of these transporters was remarkably elevated in human cancers [14,21]. Moreover, MET is a better substrate for ASCT2 and SNAT2 than phenylalanine [27,28]. These data suggest that ^{11}C-MET would be preferentially taken up in tumors with high expression of ASCT2 and/or SNAT2.

System L-specific imaging agents may be possible candidates to select patients with the therapeutic benefits of BNCT with BPA. Our results indicated that ^{18}F-FBPA is a system L-specific imaging agent. Uptake of phenylalanine derivatives such as 3-O-methyl-6-^{18}F-fluoro-L-dopa and $^{123/125}$I-2-iodo-L-phenylalanine was mediated by system L [22–24]. Wiriyasermkul et al. reported that L-3-^{18}F-α-methyl tyrosine is selectively transported by LAT1 but not by LAT2, whereas the contribution of LAT4 is unclear [29].

Based on our results, ^{18}F-FBPA is a selective substrate for system L containing LAT1 and LAT4 in three human glioblastoma cell lines. Other amino acid transporters such as system ASC and system A partly contributed to the ^{14}C-MET uptake compared with ^{18}F-FBPA. Further studies are needed to investigate the correlation between the tracer uptake and BPA contents in tumor tissues for selecting patients who may benefit from BNCT with BPA.

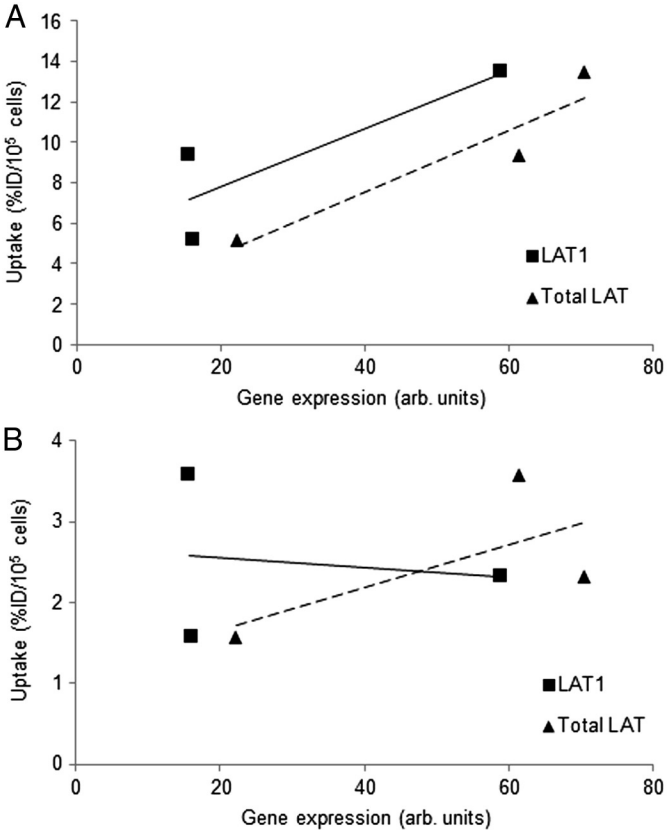

Fig. 3. Correlation between tumor uptake of ^{18}F-FBPA (A) and ^{14}C-MET (B) and the expression of amino acid transport system genes. ^{18}F-FBPA positively correlated with LAT1 and total LAT ($r = 0.8576$, $p = 0.3440$ for LAT1 and $r = 0.9418$, $p = 0.2183$ for total LAT). Although there was no correlation between ^{14}C-MET uptake and LAT1 ($r = -0.1497$, $p = 0.9043$), total LAT had a weak correlation with ^{14}C-MET ($r = 0.6691$, $p = 0.5334$).

Acknowledgments

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