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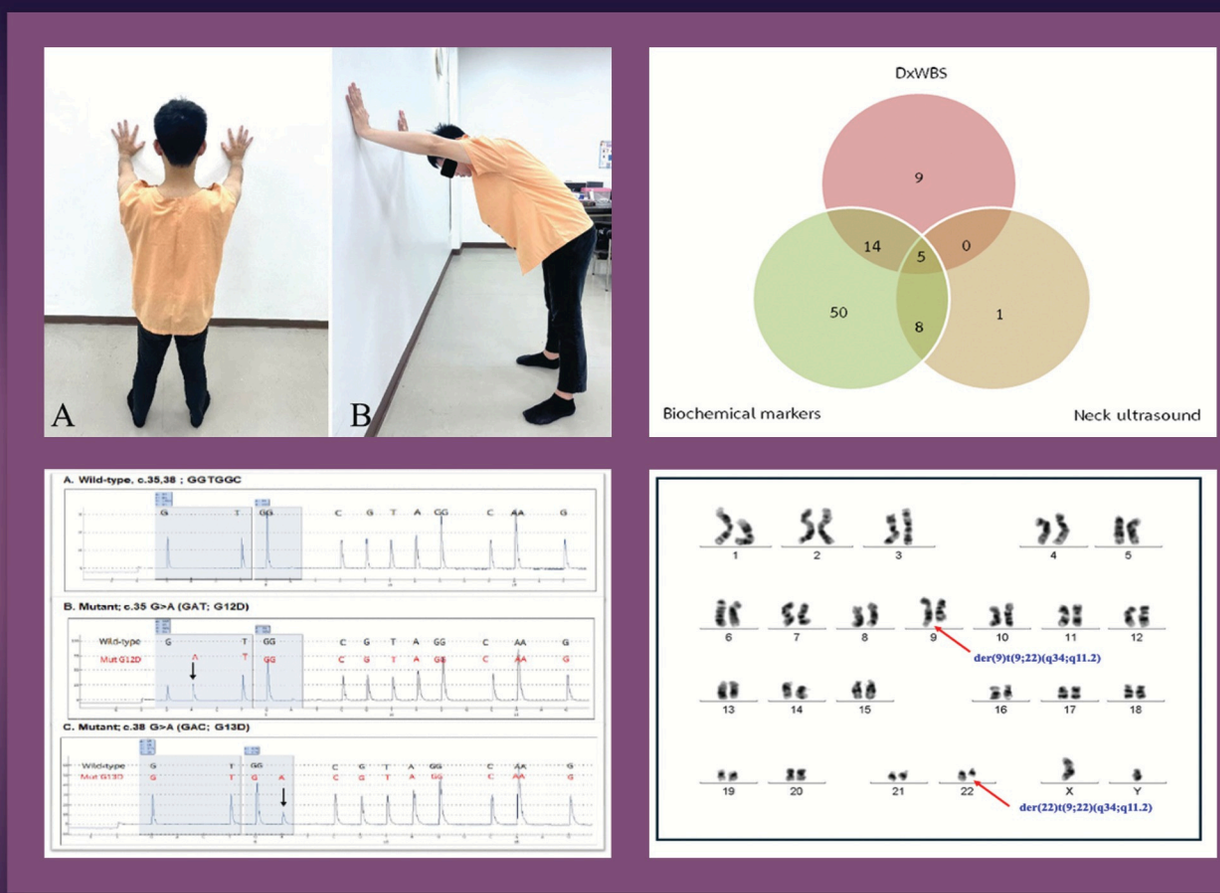

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Knowledge towards exercise and foot care among individuals with uncontrolled type 2 diabetes mellitus in secondary care settings in northeastern Thailand: a cross-sectional study

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KEYWORDS
Non-communicable disease; Community healthcare service; Diabetic foot care; Out-patient diabetes knowledge; Physical activity.

ABSTRACT
Type 2 diabetes mellitus (T2DM) poses a global public health concern due to complications arising from insufficient knowledge of self-care practices. Despite the significant impact on blood glucose control and the prevention of foot amputation, research on the knowledge of exercise and foot care among individuals with uncontrolled T2DM in northeastern Thailand is lacking. Accurate comprehension of foot care and exercise knowledge is essential for these individuals to avoid inefficacy and potential harm. The objective of this study was to investigate knowledge toward exercise and foot care among individuals with uncontrolled T2DM at secondary care setting in northeastern Thailand. Data were gathered through a self-developed questionnaire with high internal consistency (KR-20=0.87) distributed to 170 uncontrolled T2DM patients residing in the Warichaphum district, Sakon Nakhon province. Descriptive statistics were employed for data analysis. Among these respondents, 58.82% demonstrated a 'Good' level of knowledge regarding exercise, while 85.71% showed a 'Good' level of knowledge regarding foot care. Nonetheless, respondents provided incorrect answers to several key items, highlighting the need to correct misconceptions for better comprehension. This study offers valuable insights for designing and implementing initiatives aimed at enhancing knowledge and promoting better exercise habits for blood sugar control among uncontrolled T2DM patients.

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Introduction

Diabetes mellitus (DM) is a chronic non-communicable disease that has a detrimental impact on public health systems worldwide. There are over 422 million people with diabetes globally, and 1.5 million died from it[1]. In Thailand, the incidence of type 2 diabetes mellitus (T2DM) was 4.8 million people in 2019 but only 35.6% received a proper diagnosis and treatment[2]. According to the mentioned reason, the average number of diabetes deaths reached 200 per day[2]. Diabetes is the leading cause of cardiovascular disease, neuropathy, nephropathy, and retinopathy[3]. Patients with type 2 diabetes in the northeast region showed lower glycemic control than those in other regions, possibly influenced by health system and personal factors[4]. Further, the prevalence of glutinous rice consumption, a high-carbohydrate staple in this region, contributes to the higher risk of hypertriglyceridemia compared to non-glutinous rice in other regions[5]. Additionally, the northeast region had the lowest physician-to-population ratio relative to the rest of the country, impacting diabetes care outcomes[6]. Hence, depending solely on data from other regions may overlook the complexities and unique characteristics specific to the northeastern population. Factors such as cultural practices, dietary habits, socioeconomic status, and prevalence of certain health conditions may vary between regions, making it essential to gather region-specific data for a comprehensive understanding. Based on the 2019 medical statistics report from Waritchaphum Hospital in Sakon Nakhon province, the prevalence of diabetes was documented as 2,554 cases, with 303 cases classified as uncontrolled T2DM.

Regardless of age, various types of physical activity have the potential to substantially enhance the well-being and glycemic regulation of people with T2DM[7]. This consists of flexibility, balance exercises, as well as aerobic and muscle-strengthening exercises[7]. Implementing precautions is crucial for ensuring the safe and effective engagement of T2DM patients with conditions such as diabetic retinopathy, autonomic neuropathy, peripheral neuropathy, or diabetic kidney disease in physical activity[7].

Diabetic patients suffer major lifestyle impact from foot amputation[8]. While self-care management positively influences glycemic control and diabetic foot care complications[9], diabetes remains responsible for over 70% of global diabetic foot loss[10]. In Thailand, foot ulcers have a prevalence of 1-20%[11], with foot amputations occurring at a rate of 1.5%[12]. These statistics emphasize the importance of imparting diabetes knowledge to lower complication rates.

The clinical practice guidelines for diabetes 2017[13] were established by the Diabetes Association of Thailand under the patronage of Her Royal Highness Princess Maha Chakri Sirindhorn, the Endocrine Society of Thailand, and the Institute of Medical Research and Technology Assessment, Department of Medical Services, Ministry of Public Health. The guidelines aim to enhance the provision of high-quality diabetes care services for healthcare professionals in Thailand. However, there is a shortage of data regarding the knowledge towards exercise and foot care among individuals with uncontrolled T2DM in secondary care settings in northeastern Thailand, as stipulated by the guidelines. A correct understanding of knowledge on exercise and foot care is essential for individuals with uncontrolled T2DM to prevent complications such as cardiovascular disease, neuropathy, nephropathy, and foot amputation. Hence, the objective of this study was to investigate the knowledge of exercise and foot care in accordance with the clinical practice guidelines for diabetes 2017, among individuals with uncontrolled diabetes (fasting blood sugar [FBS] ≥ 183 mg/dL)[14].

Materials and methods

Study population, sample size and sampling

A cross-sectional study was done among uncontrolled T2DM patients attending diabetic clinic at Waritchaphum Hospital, Sakon Nakhon province, Thailand to assess their knowledge

towards exercise and foot care. We used a single proportion formula[15] to estimate the minimum require sample size given as $n = [NZ^2_{\alpha/2}P(1-P)]/[e^2(N-1)+Z^2_{\alpha/2}P(1-P)]$, where n is the minimum required sample size, N is the number of uncontrolled T2DM patients at the hospital (303 cases), and P is the estimated proportion of knowledge towards exercise among diabetic patients (49.5%) based on the previous study[16]. Additionally, e is the margin of error (5%), and Z is the standard normal value (1.96) corresponding to a 95% CI with a significance level = 95%, *p*-value = 0.44, N = 303, and d = 0.05, the estimated number of respondents was 170. The inclusion criteria were people with T2DM who (1) have been diagnosed with T2DM for ≥ one year, (2) were 35 years of age and above, (3) were uncontrolled diabetes FBS ≥ 183 mg/dl[14], (4) residing in that locality for more than a year, and (5) could read and answer questionnaire in Thai. Exclusion criteria were individuals who (1) have been diagnosed with type 1 diabetes mellitus or gestational diabetes mellitus and (2) have foot ulcer or foot amputation. The study participants were selected using a systematic random sampling technique until the intended number was obtained. Prior to data collection, ethical approval was obtained from the Khon Kaen University Ethics Committee for Human Research (HE632253). Individuals meeting the study criteria were enrolled after providing informed consent.

Data collection

Data collection took place at Waritchaphum Hospital in Sakon Nakhon province. The principal investigator sought and obtained permission from the hospital director for data collection. A trained research assistant distributed questionnaires to randomly selected 170 uncontrolled T2DM patients. Participants voluntarily completed the questionnaire, taking approximately 15 minutes, and deposited it in the box at the diabetes clinic in Waritchaphum Hospital. The research assistant subsequently reviewed the questionnaires for completeness.

Instrument and study variables

The current study employed a self-report questionnaire (Supplementary part) developed in accordance with the clinical practice guidelines for diabetes 2017[13]. This questionnaire underwent assessments of both its content validity and reliability through the following procedures. Three certified physiotherapists with over twenty years of experience evaluating the content in terms of its accuracy, appropriateness, and clarity of language and questions. Subsequently, the researchers refined the questionnaire based on the feedback from these experts. The revised questionnaire with an internal consistency reliability of 0.87, as determined by the Kuder-Richardson 20 method[17] was applied to 10 patients with uncontrolled T2DM who met the same inclusion and exclusion criteria and resided in a community setting that mirrored the research target area and covered a broad range of ages and educational backgrounds. The study focused on two outcome variables: knowledge of exercise and foot care. Both the exercise knowledge and foot care knowledge sections consisted of ten Yes/No questions. Each question was assigned a point value of one (1) for a correct response and zero (0) for incorrect ones. Patients demonstrate good knowledge when they answer knowledge questions with a total score of seven or higher. On the other hand, patients are considered to have poor knowledge if their mean score for knowledge questions is less than seven[18,19].

Data analysis

IBM SPSS Statistics version 28.0 software (Khon Kaen University license) was used for all statistical analyses. The normality of the data was assessed using the Kolmogorov-Smirnov test, and descriptive statistics were utilized to elucidate the demographic characteristics of the participants. Categorical variables were presented as frequencies and percentages. Continuous variables were described with means and standard deviations for normally distributed data, or with medians and interquartile ranges for non-normally distributed data.

Results

Participants' characteristics

This cross-sectional study initially involved 170 patients with uncontrolled T2DM. However, due to various reasons, including incomplete response (23 participants) and failure to return the questionnaire (28 participants), a total of 51 participants were subsequently excluded from the analysis. Ultimately, the study obtained 119 valid responses, yielding a response rate of 70%. The demographic details are presented in table 1.

Table 1 Demographic characteristics of the participants

Characteristics	Overall (n = 119)	Male (n = 23)	Female (n = 96)
Age (year)			
Mean±SD	62.04±9.27	62.48±10.04	61.94±9.13
95% CI	60.36–63.72	58.14–66.82	60.09–63.79
Fasting blood sugar (mg/dL)			
Mean±SD	200.03±10.80	198.70±10.68	200.34±10.86
95% CI	198.06–201.99	194.08–203.31	198.14–202.54
Duration of diabetes (year)			
1-10 years [n (%)]	92 (77.31)	23 (100)	69 (71.87)
11-20 years [n (%)]	21 (17.65)	0 (0)	21 (21.88)
21-30 years [n (%)]	6 (5.04)	0 (0)	6 (6.25)
Mean±SD	8.01±6.23	5.59±2.54	8.59±6.71
95% CI	6.88–9.14	4.49–6.69	7.24–9.95
Educational level [n (%)]			
Primary school	103 (86.56)	17 (73.91)	86 (89.58)
High school	13 (10.92)	5 (21.74)	8 (8.34)
Bachelor or advance degree	2 (1.68)	1 (4.35)	1 (1.04)
Other (vocational certificate)	1 (0.84)	0 (0.00)	1 (1.04)
Marital status [n (%)]			
Married	93 (78.15)	21 (91.30)	72 (75.00)
Unmarried	26 (21.85)	2 (8.70)	24 (25.00)
Occupation [n (%)]			
Civil servants	3 (2.52)	1 (4.35)	2 (2.08)
Business	9 (7.57)	1 (4.35)	8 (8.34)
Agriculture	93 (78.15)	15 (65.21)	78 (81.25)
Unemployed (housewife, retired)	14 (11.76)	6 (26.09)	8 (8.33)
Other comorbidities [n (%)]			
No	73 (61.34)	17 (73.91)	56 (58.33)
Yes (HT, HLD, PUD, BA)	46 (38.66)	6 (26.09)	40 (41.67)

Abbreviation: SD, standard deviation; CI, confidence interval; HT, hypertension; HLD, hyperlipidemia; PUD, peptic ulcer disease; BA, bronchial asthma.

Knowledge of exercise among individuals with uncontrolled T2DM

The highest accuracy in responding to items related to physical exercise knowledge among individuals with type 2 diabetes was observed for Item 9, followed by Item 5, Item 1, Item 3, and Item 6. Conversely, Item 8 garnered the highest percentage of incorrect responses within the sample. Additionally, a balanced distribution of correct and incorrect responses was noted for Item 4 and Item 10, with approximately 50% accuracy. Although a majority of the sample accurately responded to Items 2 and 7, a significant proportion of 33.6% and 37.8%, respectively, provided incorrect answers to these items (Table 2).

Table 2 Knowledge of exercise among individuals with uncontrolled T2DM (n=119)

Items	Correct responses n (%)	Knowledge tier		Knowledge score median [IQR1-3]
		Good n (%)	Poor n (%)	
1. After eating, taking a 10-minute walk can assist in reducing blood sugar levels (✓)	98 (82.4)	70 (58.82)	49 (41.18)	7 [6-8]
2. Engaging in exercise is not advised for individuals with a blood sugar level exceeding 300 mg/dL (✓)	79 (66.4)			
3. If your blood sugar is under 100 mg/dL, it's advisable to have a snack or hard candies before you start exercising (✓)	95 (79.8)			
4. If you have kidney impairment due to diabetes, it is strictly prohibited to engage in any form of exercise (×)	71 (51.7)			
5. If you experience left-sided chest pain, palpitations, or unusual fatigue, it is crucial to undergo an exercise stress test before engaging in any exercise (✓)	99 (83.2)			
6. Engaging in a minimum of 150 minutes of moderate intensity exercise each week, where you can talk comfortably and finish sentences without getting breathless, can help reduce blood sugar levels (✓)	94 (79.0)			
7. Engaging in a minimum of 75 minutes of vigorous intensity exercise each week, where you may need to pause for breath every few words, can help reduce blood sugar levels (✓)	74 (62.2)			
8. Checking your blood sugar level before exercising is not necessary (×)	36 (30.3)			
9. Diabetic patients should include resistance exercise at least two days a week along with aerobic exercise for 3 to 5 days a week (✓)	102 (85.7)			
10. Diabetic patients with retinopathy complications should avoid strenuous exercise (✓)	61 (51.3)			

Note: Good knowledge, total score ≥ 7; Poor knowledge, total score < 7.
Abbreviation: IQR, interquartile range.

Knowledge of foot care among individuals with uncontrolled T2DM

The highest accuracy in responding to items related to foot care knowledge among individuals with type 2 diabetes was observed for Item 9, followed by Item 6. Conversely, Item 10 garnered the highest percentage of incorrect responses within the participants, followed by Item 2, Item 5, and Item 3. While most of the participants correctly answered Item 1, Item 4, Item 7, and Item 8, a substantial percentage of 35.3%, 35.3%, 38.7%, and 41.2%, respectively, exhibited inaccuracies in their answers to these items (Table 3).

Table 3 Knowledge of foot care among individuals with uncontrolled T2DM (n=119)

Items	Correct response n (%)	Knowledge tier		Knowledge score median [IQR1-3]
		Good n (%)	Poor n (%)	
1. Every diabetic patient should undergo a thorough foot examination by a physician at least once a year (✓)	77 (64.7)	102 (85.71)	17 (14.29)	5 [4-6]
2. You should moisturize your feet, particularly skin bridging the toes (×)	25 (21.0)			
3. If you experience a decreased sensation in your feet, it is advisable to wear a toe-post sandal (×)	37 (31.1)			
4. Try not to walk barefoot even in your house (✓)	77 (64.7)			
5. To avoid infections, cut your nails short and round the corners (×)	28 (23.5)			
6. You should avoid crossing your legs as it may compress the nerves beneath the knee joint (✓)	84 (70.6)			
7. Try not to wear sock, because they might moist your foot until the wound occurs (×)	73 (61.3)			
8. To get rid of the corns or calluses on your foot, you can scrape, trim, or peel it off by yourself (×)	70 (58.8)			
9. If you have a small wound on your foot, clean it twice a day with sterile normal saline. Avoid using alcohol, betadine, Dakin solution, or hydrogen peroxide for wound care (✓)	106 (89.1)			
10. If you experience a loss of sensation in your feet, it's advisable to soak them in hot water to stimulate sensory perception (×)	13 (10.9)			

Note: Good knowledge, total score ≥ 7; Poor knowledge, total score < 7.
Abbreviation: IQR, interquartile range.

Discussion

Investigating knowledge of exercise and foot care in individuals with uncontrolled T2DM is crucial for promoting optimal self-management practices, preventing complications, addressing healthcare disparities, and ultimately improving the overall quality of life for individuals living with this chronic condition. The objective of this study was to investigate the knowledge of exercise and foot care in accordance with the clinical practice guidelines for diabetes 2017[13] among 170 individuals with uncontrolled T2DM in secondary care settings in northeastern Thailand. To improve the study's generalizability to individuals with uncontrolled T2DM in the northeastern, several steps were taken. This included incorporating a representative sample from diverse demographic backgrounds and employing rigorous research methods such as systematic random sampling and standardized data collection. We had 119 valid responses, yielding a response rate of 70%, which exceeds the generally accepted 60% response rate for survey research[20]. Our findings revealed that 58.82% had a 'Good' level of knowledge on exercise, and 85.71% showed a 'Good' level of knowledge on foot care. However, incorrect responses to key items suggest a need for addressing misconceptions to improve overall understanding.

The higher number of female participants may be associated with increased insulin resistance post-menopause and with older age[21]. In our study 58.82% of individuals with uncontrolled T2DM exhibited a 'Good' level of knowledge regarding exercise, which aligns with the findings reported by Awotidebe et al[16]. The researchers found that 49.5% of individuals with T2DM had a good understanding of exercise's impact on controlling plasma blood glucose levels. Nevertheless, 90.0% showed a negative attitude towards physical activity. In contrast, Mwimo et al[22] found that diabetes patients had a greater level of knowledge (98.4%) and positive attitudes (95.6%) towards exercise compared to our study. This difference was largely attributed to the main contributing factor of a healthcare provider's or doctor's recommendation (96%) regarding the advantages of physical activity for individuals with diabetes.

The severe complications associated with diabetes, encompassing both macrovascular issues such as cardiovascular disease, stroke, and peripheral vascular disease, and microvascular problems like nephropathy, retinopathy, and neuropathy, contribute to elevated mortality rates and a general deterioration in the quality of life among individuals with uncontrolled T2DM[23,24]. Consequently, a lack of accurate understanding regarding the role of exercise in regulating blood sugar levels could not only be ineffective but also potentially harmful for individuals with uncontrolled T2DM.

According to the exercise knowledge, up to 41.18% of all respondents had a 'Poor' level of knowledge in our study. The respondent provided inaccurate answers to several crucial Items (8, 10, and 4). Particularly, inaccurate understanding of Item 8, which states that "Checking your blood sugar level before exercising is not necessary" can have negative consequences for individuals with uncontrolled T2DM. This practice functions as a preventative measure to mitigate the potential complications of hyperglycemia and hypoglycemia. Hence, while the participants may possess knowledge regarding the role of exercise on blood sugar control, any misconceptions must be rectified to promote a more precise comprehension.

Diabetic foot ulceration (DFU) can cause disability, death, and recurrent hospitalizations[25]. Lifetime incidence in diabetics is 15%–25%[26], with 65% re-ulceration after five years[27]. DFU management can be complicated by peripheral artery disease, infection, and neuropathy, which may need amputation[27,28]. People with DFU-related amputations have lower quality of life[29] and more psychological distress[30]. In our study, 14.29% of respondents demonstrated a 'Poor' level of foot care knowledge. In line with the findings of Mahatthanapradir et al[31], who examined foot care knowledge among 440 patients at Ku Kaew Hospital

in Udon Thani province, it was revealed that over 80.9% possessed a high level of diabetic knowledge. However, 19.1% exhibited misunderstanding regarding peripheral artery intractable lesions and numbness, leading to improper foot care and heightened foot complication risks. The findings suggest a necessity to raise awareness about foot care among diabetic patients in the community, aligning with the clinical practice guidelines for diabetes 2017. Results from the current study on foot care knowledge revealed significant inaccuracies in responses, particularly for Items 2, 3, 5, and 10. Especially Item 10, which states that "If you experience a loss of sensation in your feet, it's advisable to soak them in hot water to stimulate sensory perception", it is important to emphasize that individuals with T2DM and diminished foot feeling should avoid bathing their feet in hot water to improve sensory perception. When there is a decrease in the capacity to feel sensations in the feet, immersing the feet in hot water not only fails to stimulate the patient's sensory receptors, but also increases the risk of unintentionally developing blisters due to the difficulty in detecting excessive heat[32].

The possible explanations for the high rates of incorrect responses to these specific items may include the absence of these topics in the diabetes educational program provided by the physical therapist, as well as their omission from the knowledge pamphlets distributed to all patients diagnosed with T2DM. Public health authorities should play a key role in managing patients with T2DM. There is a pressing need to raise awareness about exercise in diabetes, adhering to the guidelines[13]. This should extend to a broader dissemination of information through various channels such as radio, television, community news towers, and social media, considering their significant impact on people's behavior, including the elderly who have widespread access to these platforms[33,34].

This study is limited in exploring attitudes and practices related to exercise and foot care as outlined in the guidelines[13]. A suggestion for further study could be to investigate the attitudes and practices concerning exercise and foot care among individuals with uncontrolled T2DM. This could provide valuable insights into the factors influencing behavior change and adherence to recommended healthcare practices in this population. Nevertheless, this study offers initial data that can be utilized by public health organizations responsible for managing patients with T2DM to gain insights into exercise and foot care, in accordance with the guidelines[13]. Furthermore, it can serve as a foundation for subsequent investigations.

Conclusion

This study investigated the knowledge exercise and foot care based on the clinical practice guidelines for diabetes 2017[13] among individuals with uncontrolled diabetes at Waritchaphum Hospital, Sakon Nakhon province. Among these respondents, 58.82% demonstrated a 'Good' level of knowledge regarding exercise, while 85.71% showed a 'Good' level of knowledge regarding foot care. Nonetheless, respondents provided incorrect answers to several key items, highlighting the need to correct misconceptions for better comprehension.

Clinical implication

This study offers key insights for enhancing exercise and foot care knowledge in uncontrolled T2DM patients. It underscores the necessity of correcting misconceptions about exercise and foot care for better comprehension among this group. Accurate understanding the knowledge of foot care and exercise is crucial for individuals with uncontrolled T2DM, as it can prevent ineffectiveness and potential harm.

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary

Self-report questionnaire:
Knowledge towards exercise and foot care among individuals with uncontrolled type 2 diabetes mellitus in secondary care settings in northeastern Thailand

Part 1: General information of respondent

Please answer a few general questions about yourself by circling in the correct answer or by filling in the space provided.

1.1 What is your gender?	1. Male 2. Female
1.2 What is your date of birth?	_____/_____/_____
1.3 What is the duration since your type 2 diabetes diagnosis?	_____ years
1.4 What is your most recent fasting blood glucose level?	_____ mg/dL
1.5 What is the highest education you received?	1. Primary school 2. High school 3. Bachelor's degree or higher 4. Vocational certificate
1.6 What is your marital status	1. Married 2. Unmarried
1.7 What is your current professional occupation?	1. Civil servants 2. Business 3. Agriculture 4. Unemployed
1.8 Do you have any other underlying medical conditions?	1. NO 2. Yes, please specify_____

Part 2: Knowledge of exercise in the management of diabetes mellitus

Please complete the True or False questionnaire consisting of 10 items by indicating your response with a circle around the appropriate choice for each statement.

Items	**Answer**	
1. After eating, taking a 10-minute walk can assist in reducing blood sugar levels	True	False
2. Engaging in exercise is not advised for individuals with a blood sugar level exceeding 300 mg/dL	True	False
3. If your blood sugar is under 100 mg/dL, it's advisable to have a snack or hard candies before you start exercising	True	False
4. If you have kidney impairment due to diabetes, it is strictly prohibited to engage in any form of exercise	True	False
5. If you experience left-sided chest pain, palpitations, or unusual fatigue, it is crucial to undergo an exercise stress test before engaging in any exercise	True	False
6. Engaging in a minimum of 150 minutes of moderate intensity exercise each week, where you can talk comfortably and finish sentences without getting breathless, can help reduce blood sugar levels	True	False
7. Engaging in a minimum of 75 minutes of vigorous intensity exercise each week, where you may need to pause for breath every few words, can help reduce blood sugar levels	True	False
8. Checking your blood sugar level before exercising is not necessary	True	False
9. Diabetic patients should include resistance exercise at least two days a week along with aerobic exercise for 3 to 5 days a week	True	False
10. Diabetic patients with retinopathy complications should avoid strenuous exercise	True	False

Part 3: Knowledge of foot care in the management of diabetes mellitus

Please complete the True or False questionnaire consisting of 10 items by indicating your response with a circle around the appropriate choice for each statement.

Items	**Answer**	
1. Every diabetic patient should undergo a thorough foot examination by a physician at least once a year	True	False
2. You should moisturize your feet, particularly skin bridging the toes	True	False
3. If you experience a decreased sensation in your feet, it is advisable to wear a toe-post sandal	True	False
4. Try not to walk barefoot even in your house	True	False
5. To avoid infections, cut your nails short and round the corners	True	False
6. You should avoid crossing your legs as it may compress the nerves beneath the knee joint	True	False
7. Try not to wear sock, because they might moist your foot until the wound occurs	True	False
8. To get rid of the corns or calluses on your foot, you can scrape, trim, or peel it off by yourself	True	False
9. If you have a small wound on your foot, clean it twice a day with sterile normal saline. Avoid using alcohol, betadine, Dakin solution, or hydrogen peroxide for wound care	True	False
10. If you experience a loss of sensation in your feet, it's advisable to soak them in hot water to stimulate sensory perception	True	False

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Development of a Multiplex Allele-Specific Real-time Polymerase Chain Reaction Assay for detection of KRAS gene mutations in Thai colorectal cancer tissues

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KEYWORDS
Multiplex Allele-Specific Real-time PCR Assay; KRAS gene mutations; Colorectal cancer tissues.

ABSTRACT
Mutation analysis of KRAS is necessary before starting treatment with monoclonal anti-EGFR antibodies for effective and appropriate treatment for individual patients. The objective of this study is to develop a Multiplex Allele-Specific Real-time PCR assay for analysis of the mutational status of KRAS codons 12 and 13, including 7 types of KRAS mutations (G12D, G12A, G12R, G12C, G12S, G12V, and G13D). 160 of FFPE colorectal cancer tissues were collected from Department of Medical Services, Institute of Pathology. DNA was isolated from the FFPE tissue using AS-primers specific for mutant DNA. Moreover, the MAS-Real-time PCR analysis of samples showed good concordance (K=0.837, 95% CI 0.740-0.933) with pyrosequencing. In addition, the MAS-Real-time assay has a sensitivity of 78.26% and specificity of 100%. Our developed MAS-Real-time PCR can be applied for detection of KRAS gene mutations in FFPE tissues which is a reliable, rapid, cost-effective method and not requiring advanced instruments.

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Introduction

Colorectal cancer (CRC) is the most prevalent cancer worldwide and ranks as the third leading cause of cancer-related deaths[1]. In Thailand, CRC is the third most common cancer among men and the fifth most common among women[2]. Mutations in the KRAS gene lead to the activation of the RAS protein, promoting increased cell division, inhibition of apoptosis, induction of angiogenesis, and enhanced metastasis[3]. Monoclonal antibodies like cetuximab and panitumumab have been developed for cancer treatment, targeting the EGFR[4]. These antibodies aim to inhibit EGFR tyrosine kinase activation and downstream signaling[5]. However, these treatments are effective only for CRC with a wild-type KRAS proto-oncogene, with no response in cases with KRAS mutations[6]. Therefore, detecting KRAS gene mutations is crucial for tailoring personalized therapeutic strategies for patients[4].

Various molecular methods have been developed to detect KRAS mutations, including direct sequencing[6], pyrosequencing[7,8], co-amplification at lower denaturation temperature PCR[9], and digital PCR[10]. Additionally, commercial molecular kits are available for KRAS mutation detection, such as the cobas® KRAS Mutation Test[11], therascreen® KRAS RGQ PCR Kit[12], and KRAS PyroMark Q96 V2.0 Kit[13]. However, these methods require specialized equipment and expertise, making them costly for cancer patient prognosis and diagnosis. In contrast, Multiplex Allele-Specific Polymerase Chain Reaction (MAS-PCR) is an economical, reliable method for detecting known mutations and single-nucleotide polymorphisms[14]. Real-time PCR methods for SNP detection are increasingly important for genotyping variations in genomes[15]. MAS-Real-time PCR uses primers with allele-specific 3' termini that specifically target mutated DNA templates[14,16].

In this study, we developed a MAS-Real-time PCR assay to analyze mutations in KRAS codons 12 and 13. The most frequent single nucleotide point mutations in the KRAS gene are found in codons 12 and 13, accounting for 80 to 82% of the mutations[17-19]. The most frequent point mutations in codons 12 and 13, G12D, G12A, G12R, G12C, G12S, G12V, and G13D[8,19,20], were found to be present in formalin-fixed, paraffin-embedded tissue samples from 160 patients with colorectal cancer. The MAS-Real-time PCR method for FFPE samples was devised due to the efficient preservation of cellular, architectural, and morphological details in formalin-fixed paraffin-embedded (FFPE) tissues, which can be conveniently stored at room temperature for long durations, thus streamlining handling and storage processes[7,11]. However, FFPE processing can degrade DNA extraction efficacy and quality, posing challenges to conducting precise molecular analyses and potentially impacting the accuracy of KRAS analysis results. Furthermore, in the future, researchers plan to develop MAS-Real-time PCR for analyzing KRAS mutations from cfDNA samples. This is because there is a growing preference for detecting cancer gene mutations from cfDNA samples obtained through liquid biopsy[9,10], rather than from solid tissue or FFPE DNA samples. Pyrosequencing, a well-established and sensitive method, served as the reference for evaluating the sensitivity of the MAS-Real-time PCR assay in detecting KRAS mutant alleles.

Materials and methods

Clinical samples

Formalin-fixed, paraffin-embedded colorectal adenocarcinomas from 160 patients with CRC were collected from the Institute of Pathology, Ministry of Public Health, Bangkok, Thailand. The study was approved by the Ethics Committee of the Institute of Pathology (IOP-KM-R64-002). The pathologist reexamined the histomorphology of all samples to review and mark the adenocarcinoma areas of the hematoxylin and eosin stained slides.

DNA extraction of FFPE tissues

Each tissue sample was manually micro-dissected from areas containing only cancerous cells within paraffin-embedded blocks. Ten µm thick ribbon sections were put in a microcentrifuge tube. Paraffin was removed from the tissue sections with xylene and rehydration with 100% ethanol, and samples were air-dried. DNA was extracted from FFPE tissues and purified using a QIAamp DNA FFPE Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. DNA quantity was determined by NanoDrop spectrophotometry (NanoDrop Technologies, Wilmington, DE).

PCR amplification and pyrosequencing

Pyrosequencing for analysis of a KRAS gene fragment spanning codons 12 and 13 was performed as previously described by Ogino et al[8]. Reactions and PCR conditions were performed as previously described by Seekhuntod et al[19]. PCR products were confirmed by 8% polyacrylamide gel electrophoresis, and gels were stained with SYBR Green I Nucleic Acid Gel Stain (1:400, Lonza, USA). PCR products in 30 µl were mixed with 3 µl streptavidin-conjugated Sepharose beads (Amersham Biosciences AB, Sweden), 40 µl binding buffer, and 17 µl distilled water. The mixture was shaken at 1400 rpm for 10 min. The biotinylated PCR products were captured using a vacuum prep tool. Single-stranded DNA purification involved washing the vacuum prep tool with 70% ethanol for 5 s, denaturation solution for 5 s, and washing buffer for 10 s. Biotinylated single-stranded DNA was introduced into a 96-well microtiter plate, which contained 40 µl of a 0.4 µM solution of sequencing PF1-primer (5'-TGTGGTAGTTG-GAGCTG-3') for analyzing positions 35 and 38 in the nucleotide sequence, along with PF2-primer (5'-TGTGGTAGTTGGAGCT-3') for analyzing position 34[8]. Afterward, the plate was incubated at 80°C for 2 minutes, followed by a 5-minute cooling period to room temperature, and then loaded onto the PyroMark Q96 ID system (Qiagen, Germany), as shown in supplementary figure S1.

DNA cloning

Genomic DNA from eight clinical samples containing KRAS wild-type DNA and seven KRAS codon 12 and 13 point mutations (G12D, G12A, G12R, G12C, G12S, G12V, and G13D) underwent PCR amplification using universal KRAS primers (KRAS-codon 12/13-F and KRAS-codon 12/13-R) were performed as previously described by Seekhuntod et al[19]. The resulting 259-bp PCR products were cloned into the psc-A-amp/kan vector, transformed into competent Escherichia coli cells using a Strataclone PCR cloning kit from Agilent Technologies (USA). Transformed bacteria were plated on selective LB-agar plates with ampicillin and X-Gal and incubated at 37°C overnight. White colonies were randomly selected and cultured in LB medium overnight. Plasmids were extracted using the Wizard® genomic DNA purification kit from Geneaid (Taipei, Taiwan) and screened for the insert fragment through PCR. Positive PCR products were sequenced by the Bioneer Corporation in Daejeon, Republic of Korea.

Primer and Probe Design

Allele-specific (AS) primers were custom-designed for seven distinct mutations, each tailored to the specific mutation. A mutation-unspecific segment was used as a reference amplicon. The 3′ terminal base of each AS primer was selected according to its corresponding mutation. Amplification reactions involved the primary KRAS forward primer and five AS primers (G12R-F, G12C-F, G12D-F, G12A-F, and G13D-F), which shared one common antisense KRAS reverse primer. Additionally, reactions with two AS primers (G12S-R and G12V-R) shared a common sense KRAS forward primer[19]. All primers were synthesized and provided by BioDesign Co., Ltd. (BioDesign, Pathumthani, Thailand). Probes were employed to detect target amplification. Reference and allele-specific PCRs used the same probe, with opposing PCR primers. These probes were procured from Applied Biosystems, Foster City, CA. Probes for KRAS PCR quantification were labeled

with 6-fluorescein at the 5' end, with a black hole quencher™ domain at the 3' end. An exogenous internal control PCR product, a 100-base-long segment in TBXAS1 exon9, was co-amplified in both reference and allele-specific PCRs. A probe was used for internal control PCR detection, labeled with a Texas Red-fluorophore at the 5' end and featuring a black hole quencher™ domain at the 3' end. All primer and probe sequences are listed in supplementary table S1.

Multiplex Allele-Specific Real-time PCR (MAS-Real-time PCR) Assay

To perform the MAS-Real-time PCR reaction for detecting KRAS gene mutations at codons 12 and 13, this assay was designed with primers and probes specific to gene NM_004985.4, which have different PCR product sizes as shown in supplementary table S1. All reactions were carried out in 1-8 tubes, each designated for a specific mutation type: wild-type, G12S, G12R, G12C, G12D, G12A, G12V, and G13D, respectively. In each tube, the final volume was 20 µl, consisting of 10 µl of 2X KAPA probe fast qPCR master mix, 0.5 µl each of 10 µM Oligonucleotide primer Internal control-F and Internal control-R, 0.25 µl of 10 µM Oligonucleotide probe Internal control-P, and the specific AS-primer as indicated in supplementary table S2. Additionally, 2 µl of DNA template with a concentration of 20-50 ng/µl was added, and the final volume was adjusted with nuclease-free water to 20 µl. The protocol included an initial activation step at 95°C for 5 minutes, followed by an amplification step consisting of 10 cycles at 95°C for 30 seconds, 64°C for 45 seconds, and 72°C for 30 seconds. Subsequently, there were 30 cycles of amplification at 95°C for 30 seconds, 58°C for 30 seconds, and 72°C for 30 seconds. Data collection was performed during the annealing phase, with measurements in the FAM channel for KRAS gene detection. The reactions were analyzed using a Bio-RAD CFX96 real-time PCR machine for qualitative detection, with the analysis based on the amplification curve characteristics above the threshold line. The criteria for result interpretation were as follows: a ct value of < 25 in the FAM channel indicated a positive result (mutation detected), while a ct value of ≥ 25 indicated a negative result (no mutation detected). In the Texas Red channel, a ct value of < 20 indicated a valid reaction. The DNA samples used in this assay were of high quality, and the wild-type tube along with the positive 113-bp as an internal control. Wild-type DNA at any of the seven positions prevents allele-specific amplification resulting in a corresponding missing amplification curve, as shown in figure 1.

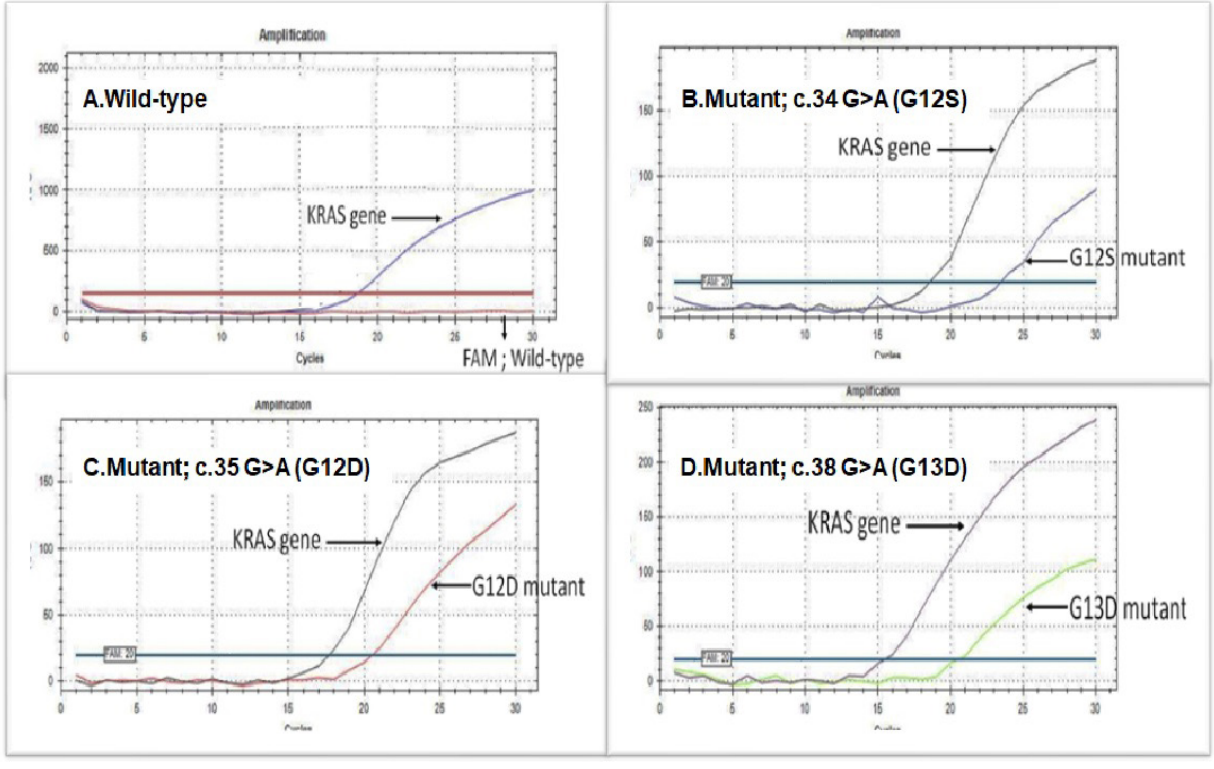

Figure 1 Genomic DNA from FFPE tissues were used for MAS-Real-time PCR assay.

(A) Wild-type nucleotide 35 by the KRAS-G12D-F primer. In red curves indicated wild-type DNA.

(B) c.34G>A (codon 12 AGT) mutation by the KRAS-G12S-R primer. In blue curves indicated G12S mutant DNA.

(C) c.35G>A (codon 12 GAT) mutation by the KRAS-G12D-F primer. In red curves indicated G12D mutant DNA.

(D) c.38G>A (codon 13 GAC) mutation by the KRAS-G13D-F primer. In green curves indicated G13D mutant DNA.

Sensitivity of MAS-Real-time PCR Assay

The eight plasmid clones containing KRAS wild-type sequences and seven plasmid clones with various KRAS mutations (G12D, G12A, G12R, G12C, G12S, G12V, or G13D) were isolated. Each mutated plasmid DNA was combined with wild-type plasmid DNA to create a total of 100 ng. In order to assess precision and reproducibility, we gradually reduced the proportion of mutant plasmid DNA, resulting in decreasing ratios of mutant to wild-type DNA at levels of 100%, 50%, 25%, 10%, 5%, 2%, 1%, and 0.1%. Precision and reproducibility were assessed through four repeated runs, with analysis conducted within the range of the lowest detection limit as determined by MAS-Real-time PCR.

Statistical analysis

The results obtained from both MAS-Real-time PCR and pyrosequencing were subject to comparison for 160 formalin-fixed, paraffin-embedded specimens. The significance of this evaluation was determined through Kappa statistics. A Kappa value greater than 0.81 was considered noteworthy, signifying that both methods produced highly accurate results. Additionally, we explored variances in categorical

variables such as age, gender, histologic grade, and tumor location among patients with KRAS mutations, employing a chi-square test. All statistical analyses were conducted as two-sided tests, with a significance threshold of *p*-value < 0.05. The analysis was carried out using SPSS software (version 19).

Results

Pyrosequencing analysis of KRAS gene mutations in CRC clinical samples

In the initial phase of the study, 160 clinical samples were analyzed through pyrosequencing, specifically targeting six distinct point mutations within codon 12 (G12S, G12R, G12C, G12D, G12A, and G12V), as well as one point mutation within codon 13 (G13D) of the KRAS gene. These mutations were selected due to their frequent occurrence in patients with colorectal cancer. Among the 160 tissue specimens, the sequencing results indicated that 46 cases (28.75%) displayed a mutation in either codon 12 or 13 of the KRAS gene, while 114 cases (71.25%) were categorized as KRAS wild type (as shown in Table 2). Of the 46 cases with a KRAS mutation, 40 (86.96%) had a mutation in codon 12. The most commonly observed codon 12 mutation was G12D, accounting for 36.96%, followed by G12V at 30.43%. The less common codon 12 mutations included G12S, G12A, G12C, and G12R, with frequencies of 8.70%, 4.35%, 4.35%, and 2.17%, respectively. The sole mutation detected in codon 13 was G13D, representing 13.04% of cases.

MAS-Real-time PCR analysis of KRAS gene mutations in CRC clinical samples

The MAS-Real-time PCR assay, performed on the 160 CRC tissue specimens to detect mutations in KRAS codon 12 and codon 13, produced results that closely corresponded with those obtained from pyrosequencing. As indicated in table 2, the MAS-Real-time PCR assay identified 36 cases (22.5%) with KRAS mutations in codons 12 and 13, while 124 cases (77.5%) were classified as KRAS wild type. More specifically, among these, 32 samples (88.89%) displayed mutations in codon 12, with the predominant mutation pattern being G12D at 36.11%, followed by G12V at 33.33%. The less common mutation patterns in codon 12 were G12S and G12R, accounting for 11.11% and 8.33%, respectively. In codon 13, only one mutation pattern, G13D, was observed in 11.11% of cases. Among the 114 FFPE DNA samples initially identified as wild-type KRAS by pyrosequencing assay, 46 samples were found to contain mutations. Within this subgroup, 27 cases (58.70%) demonstrated nucleotide transitions from G to A, followed by 16 cases (34.78%) showing nucleotide transversions from G>T, and 3 cases (6.52%) with nucleotide transversions from G>C at codon 12. The predominant mutation type observed in codon 12 was G12D (GAT), resulting in the amino acid change from glycine to asparagine, occurring in 17 cases (36.96%) among the total 46 samples, as detailed in table 1.

Table 1 KRAS mutational status in 160 CRC patients

KRAS status	Type of mutations Amino acid change	Pyrosequencing Number of mutations (%)	MAS-Real-time PCR Number of mutations (%)
Wild-type	-	114/160 (71.25%)	124/160 (77.50%)
Mutant	All Codon 12 and 13	46/160 (28.75%)	36/160 (22.50%)
Mutated Codon 12	All Codon 12	40/46 (86.96%)	32/36 (88.89%)
G12D	GGT>G<u>A</u>T Gly→Arg	17/46 (36.96%)	13/36 (36.11%)
G12V	GGT>G<u>T</u>A Gly→Val	14/46 (30.43%)	12/36 (33.33%)
G12A	GGT>G<u>C</u>T Gly→Ala	2/46 (4.35%)	0/36 ND
G12S	GGT><u>A</u>GT Gly→Ser	4/46 (8.70%)	4/36 (11.11%)
G12C	GGT><u>T</u>GT Gly→Cys	2/46 (4.35%)	0/36 ND
G12R	GGT><u>C</u>GT Gly→Arg	1/46 (2.17%)	3/36 (8.33%)
Mutated Codon 13	All Codon 13	6/46 (13.04%)	4/36 (11.11%)
G13D	GGC>G<u>A</u>C Gly→Asp	6/46 (13.04%)	4/36 (11.11%)

Note: Underlined bases represent the substitutions in the respective codon. ND denotes not detected.

Comparison of MAS-Real-time PCR and Pyrosequencing assays

The results obtained from the 160 FFPE samples were subjected to an agreement analysis, comparing the outcomes of the MAS-Real-time PCR with those from the pyrosequencing method (refer to Table 2). Among these samples, 150 out of 160 displayed matching results, indicating a high level of accuracy for both methods (p-value < 0.05), and there was no statistically significant difference between the two assays (K = 0.837, 95%CI = 0.740 to 0.933). The positive agreement was 100%, while the negative agreement was 91.94%.

Table 2 Pairwise comparison and agreement analyses between MAS-Real-time PCR and pyrosequencing assays

		Pyrosequncing		Total (%)
		Positive (%)	Negative (%)	
MAS-Real-time-PCR	Positive (%)	36 (22.50%)	0	36 (22.50%)
	Negative (%)	10 (6.25%)	114 (71.25%)	124 (77.50%)
	Total (%)	46 (28.75%)	114 (71.25%)	160 (100.00%)

Note: Kappa= 0.837, 95% confidence interval: From 0.740 to 0.933, Positive agreement: 100%, Negative agreement: 91.94%.

Examining the Association Between Patient Characteristics and Mutations in KRAS Codon 12 and 13

The median age of the patients was 64 years, with an age range spanning from 30 to 87 years. The majority of patients fell within the age group of 60 to 79 years, accounting for 60% of the total. The male-to-female ratio was 1.25 to 1. In terms of histological characteristics, the most prevalent type was moderately differentiated, comprising 68.12% of cases (109 out of 160), and the majority of cases (88.12%) were primary colorectal tumors (as detailed in Table 3). An investigation was conducted to explore potential correlations between patients' demographic attributes and the presence of detected KRAS mutations, as depicted in table 3. Among the patients with KRAS-mutated carcinomas, no statistically significant differences were identified in relation to age, gender, histologic grade, or tumor site.

Table 3 Correlation between KRAS mutation and patients' characteristics of 160 colorectal carcinomas

Characteristics	N (%)	WT KRAS N (%)	MT KRAS N (%)	*p*-value
Total patients	160			
Median age: (range)	64 years (30-87)			
20-39 years	5 (3.12%)	4 (80.00%)	1 (20.00%)	0.916
40-59 years	51 (31.88%)	36 (70.59%)	15 (29.41%)	
60-79 years	96 (60.00%)	69 (71.88%)	27 (28.12%)	
80-90 years	8 (5.00%)	5 (62.50%)	3 (37.50%)	
Gender:				
Male	89 (55.63%)	68 (76.40%)	21 (23.60%)	0.107
Female	71 (44.37%)	46 (64.79%)	25 (35.21%)	
Histologic grade:				
Well differentiated	34 (21.25%)	19 (55.88%)	15 (44.12%)	0.066
Moderate- differentiated	109 (68.12%)	81 (74.31%)	28 (25.69%)	
Poorly differentiated	17 (10.63%)	14 (82.35%)	3 (17.65%)	
Site:				
Colorectal primary	141 (88.12%)	99 (70.21%)	42 (29.79%)	
Metastasis	19 (11.88%)	15 (78.95%)	4 (21.05%)	0.430

The Sensitivity, Accuracy, and Consistency of KRAS Mutation Detection Using MAS-Real-time PCR

To assess the sensitivity of the MAS-Real-time PCR assay, plasmid DNA from each of the seven KRAS mutant clones was diluted in separated amplification reactions alongside plasmid DNA from a wild-type KRAS clone. The objective was to gradually decrease the proportion of mutant DNA to achieve lower ratios of mutant to wild-type DNA. The MAS-Real-time PCR assay successfully detected mutant alleles, reaching a sensitivity as low as 5% for the G12R, G12D, G12A, G12V, and G13D mutants, and 20% for the G12S and G12C mutants. An illustrative example of the MAS-Real-time PCR assay's lowest limit of detection can be seen in figure 2. To evaluate the precision and reproducibility of our MAS-Real-time PCR assay, we quantified KRAS mutations within DNA mixtures consisting of each of the seven mutant KRAS DNA samples and wild-type DNA at various ratios (1%, 5%, 10%, 15%, 20%, 30%, 50% and 100%) in four repeated runs. The results consistently demonstrated precision and reproducibility, consistently detecting the same lowest quantity of KRAS mutant alleles in all repeated runs.

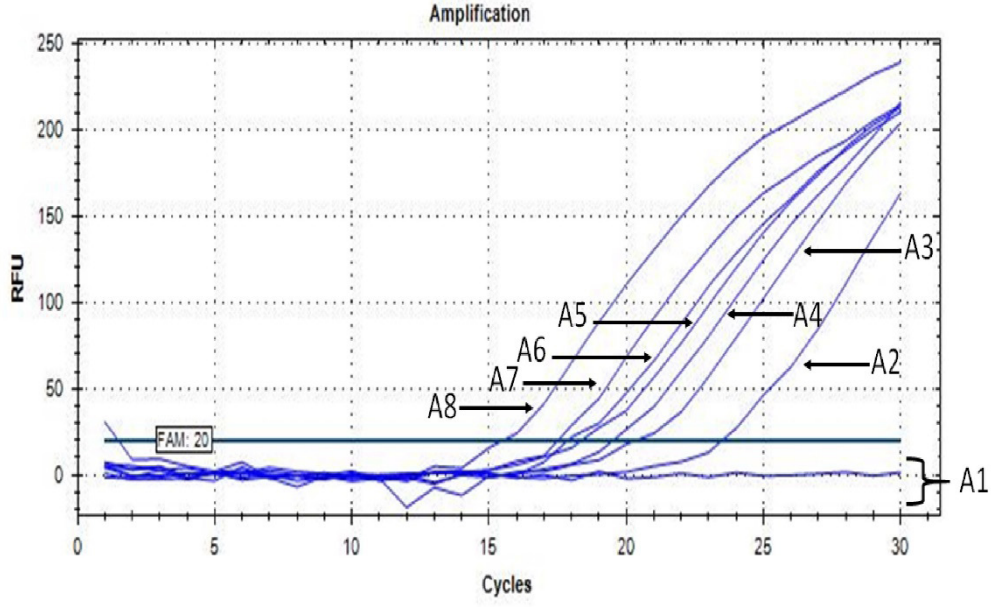

Figure 2 Sensitivity of MAS-Real-time PCR assay for identifying KRAS gene mutations.
Note: A representative amplification curve is shown. Dilutions of G12D mutant plasmid and wildtype plasmid DNA (from 1%, 5%, 10%, 15%, 20%, 30%, 50% and 100% mutated alleles). Designations A1-A8 represent DNA samples that yield positive results for the G12D mutation across varying mutation allele frequencies of 1%, 5%, 10%, 15%, 20%, 30%, 50%, and 100%, respectively.

Discussion

Colorectal cancer (CRC) ranks as the third most prevalent cancer globally and is a primary contributor to cancer-related fatalities[21]. Within Thailand, there has been a notable rise in the occurrence of CRC in recent times. Numerous studies have explored the link between KRAS mutations and colorectal cancer (CRC)[20]. These mutations in CRC patients are associated with resistance to anti-EGFR treatments like cetuximab or panitumumab[4]. Predicting therapeutic responses accurately is crucial to avoid unnecessary treatments and focus on more individualized and effective therapies. Several methods and

commercial molecular kits are available for detecting KRAS mutations[6,22,23], each with its own set of challenges. For instance, although direct sequencing is commonly used, it has low sensitivity and requires a substantial percentage (10%-30%) of mutated alleles in a wild-type background[22,24]. Pyrosequencing is accurate and feasible, with superior sensitivity (approximately 5% mutant allele)[8,22], but it involves expensive equipment and consumables, making it cost-prohibitive in developing countries. Commercial molecular kits offer advantages like high sensitivity (detection limit around 1% to < 5%), speed, easy data interpretation, and detection of various KRAS mutation positions. However, they also require costly instruments, expensive reagents, and have a relatively high cost per sample[25]. Hence, there is a pressing need to develop an accurate, simple, and cost-effective method for detecting KRAS mutations associated with CRC that can be deployed in developing countries.

In this research, we have effectively created a MAS-Real-time PCR assay that is both highly sensitive and specific, focusing on the seven most prevalent mutations (G12S, G12R, G12C, G12D, G12A, G12V, and G13D) in codons 12 and 13 of the KRAS gene. Additionally, the use of probe-based real-time PCR methods provides advantages in preventing interference between samples and/or environmental contaminants during experimental procedures.

Our findings demonstrated a high level of agreement between the MAS-Real-time PCR assay and pyrosequencing (K=0.837). However, in 10 cases, there were discrepancies in the results, particularly concerning mutations in codon 12 and codon 13. These mutations were detectable through pyrosequencing and Sanger direct sequencing but not with the MAS-Real-time PCR assay. Specifically, G12D was identified in three samples, G12V in two samples, G12A in two samples, G12C in two samples, and G13D in one sample. The inability of the MAS-Real-time PCR to detect mutations in all 10 samples may be attributed to issues such as biased amplification, primer interactions, and DNA damage resulting from the formalin fixation process during long-term storage[26,27]. This damage can lead to compromised DNA quality[28] and cross-linking with proteins, hindering the success of the MAS-PCR reaction[29,30].

Interestingly, our MAS-Real-time PCR assay exhibited a strikingly high analytical sensitivity for detection, successfully identifying approximately 5% mutant alleles in DNA mixing experiments. These experiments utilized genomic DNA isolated from plasmid cloned DNA for the G12R, G12D, G12A, G12V, and G13D mutants. Furthermore, the sensitivity of the MAS-Real-time PCR assay we developed surpasses that reported for direct sequencing and HRM (ranging from 5% to 20%), and is on par with that reported for pyrosequencing and commercial molecular kits (ranging from 1% to 5%)[8,24].

In this study, the occurrence of mutations in the KRAS gene at codons 12 and 13 was investigated in colorectal cancer patients in Thailand. A total of 160 tissue samples from colorectal cancer patients, embedded in paraffin and examined at the Institute of Pathology, Department of Medical Sciences, Ministry of Public Health, were analyzed. The findings revealed an overall mutation rate of 28.75% (46/160), with the mutation frequency ranging from 20% to 50%, closely aligned with previous research reports[19]. Mutations at codon 12 accounted for 86.96% (40/46), while those at codon 13 were found in 13.04% (6/46) of cases. The mutation rate at codon 12 ranged from 70% to 90%, while the rate at codon 13 ranged from 10% to 30%, consistent with earlier studies[18]. The most prevalent mutation types were G12D, G12V, and G13D, resembling findings from prior studies[7]. The highest mutation pattern involved the substitution of the base sequence Glycine (G) with Aspartic (A), accounting for 58.70% (27/46), in accordance with previous research[7,17].

General characteristics of colorectal cancer patients, including age, gender, histopathological features of tissue samples, and the location of cancerous masses, were also examined. It was observed that there was no significant difference in the occurrence of mutations based on age or the absence of mutations. However, some studies have reported an association between KRAS gene mutations and gender, with higher occurrences in females compared to males[6], which contradicts the findings of Poehlmann and colleagues, who reported higher occurrences in males[7].

The MAS-Real-time PCR method has a sensitivity comparable to that of direct sequencing and HRM (high-resolution melting) methods, which can detect mutant alleles in the range of 5% to 20%[8,25]. MAS-Real-time PCR is a rapid assay that can be completed in under 1.5 hours (excluding DNA isolation). It is cost-effective, with an approximate cost of $10 per test. Utilizing only a Real-time PCR instrument, the assay offers benefits in avoiding interference between samples and/or environmental contaminants during experimental procedures. Additionally, it does not demand a high level of technical expertise and specialized equipment.

Conclusion

In conclusion, we developed a MAS-Real-time PCR assay for detection of the seven most common mutations in codons 12 and 13 of the KRAS gene. MAS-Real-time PCR assay is a DNA-based protocol that was easy to perform, being rapid, cost-effective, highly sensitive and highly specific. An assay with these characteristics is important for analysis of clinical samples, such as FFPE tissues, in particular to assist clinicians in predicting the clinical course of monoclonal anti-EGFR antibody treatment of mCRC patients.

Take home messages

The MAS-Real-time PCR assay, developed here, demonstrates high sensitivity and specificity for detecting seven KRAS gene mutations (G12S, G12R, G12C, G12D, G12A, G12V, and G13D) in codons 12 and 13.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgements

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Supplementary

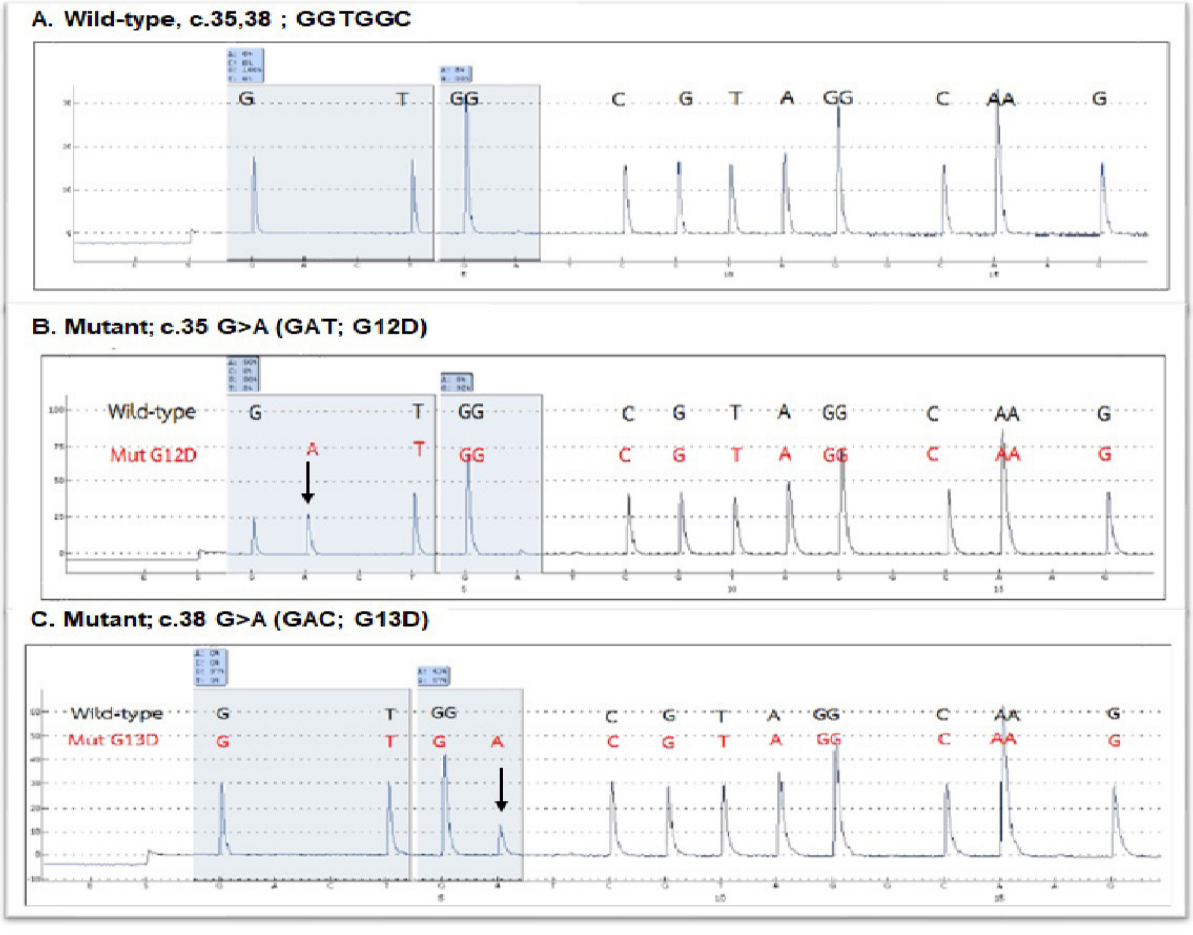

Figure S1 Pyrograms of pyrosequencing assay for identifying KRAS gene mutations.

(A) Wild-type nucleotide 35 and 38 by the KRAS-PF1 primer.

(B) c.35G>A (codon 12 GAT) mutation by the KRAS-PF1 primer.

(C) c.38G>A (codon 13 GAC) mutation by the KRAS-PF1 primer.

Note: Arrows indicate the presence of mutant alleles.

Table S1 Primers and Probe used in MAS-Real-time PCR for detecting the most common mutations in codons 12 and 13 of KRAS gene

Primer	Sequence	Sequence length (bp)	Product length (bp)
KRAS-F	5'-GGCCTGCTGAAAATGACTGAA-3'	21	113 bp
KRAS-R	5'-GGATCATATTCGTCCACAAAATG-3'	23	113 bp
KRAS-Probe	5'-FAM-TGTGGTAGTTGGAGCTGGTG-BHQ1-3'	20	-
KRAS-G12S-R	5'-CACTCTTGCCTACGCCACT-3'	19	64 bp
KRAS-G12R-F	5'-TTGTGGTAGTTGGAGCTC-3'	18	85 bp
KRAS-G12C-F	5'-CTGAATATAAACTTGTGGTAGTTGGAGCTT-3'	30	97 bp
KRAS-G12D-F	5'-ATAAACTTGTGGTAGTTGGAGCTGA-3'	25	91 bp
KRAS-G12A-F	5'-GTGGTAGTTGGAGCTGC-3'	17	83 bp
KRAS-G12V-R	5'-AAGGCACTCTTGCCTACGCCAA-3'	22	68 bp
KRAS-G13D-F	5'-AAACTTGTGGTAGTTGGAGCTGGTGA-3'	26	89 bp
Internal control-F	5'-GCCCGACATTCTGCAAGTCC-3'	20	100 bp
Internal control-R	5'-GGTGTTGCCGGGAAGGGTT-3'	19	100 bp
Internal control-Probe	5'-Texas Red-CTCCTCTACTGGGTGCAAGC-BHQ1-3'	20	-

Table S2 Details of adjusting the volume of AS-primer in the MAS-Real-time PCR reaction for KRAS gene mutations

G12S tube			G12R tube			G12C tube			G12D tube		
Primer name	**Conc.**	**Vol.**	**Primer name**	**Conc.**	**Vol.**	**Primer name**	**Conc.**	**Vol.**	**Primer name**	**Conc.**	**Vol.**
G12S-R	10 μM	0.1 μl	G12R-F	10 μM	0.65 μl	G12C-F	10 μM	0.25 μl	G12D-F	10 μM	0.15 μl
KRAS-F	10 μM	0.1 μl	KRAS-R	10 μM	0.5 μl	KRAS-R	10 μM	0.25 μl	KRAS-R	10 μM	0.4 μl
KRAS-P	10 μM	0.1 μl	KRAS-P	10 μM	0.5 μl	KRAS-P	10 μM	0.25 μl	KRAS-P	10 μM	0.2 μl
G12A tube			**G12V tube**			**G13D tube**			Wild-type tube		
Primer name	**Conc.**	**Vol.**	**Primer name**	**Conc.**	**Vol.**	**Primer name**	**Conc.**	**Vol.**	**Primer name**	**Conc.**	**Vol.**
G12A-F	10 μM	0.4 μl	G12V-R	10 μM	0.09 μl	G13D-F	10 μM	0.2 μl	KRAS-F	10 μM	0.15 μl
KRAS-R	10 μM	0.4 μl	KRAS-F	10 μM	0.09 μl	KRAS-R	10 μM	0.2 μl	KRAS-R	10 μM	0.15 μl
KRAS-P	10 μM	0.4 μl	KRAS-P	10 μM	0.09 μl	KRAS-P	10 μM	0.2 μl	KRAS-P	10 μM	0.15 μl

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ORIGINAL ARTICLE

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Role of the ^{131}I whole body scan for initial follow-up in patients with intermediate-risk differentiated thyroid cancer

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KEYWORDS
Thyroid cancer;
Intermediate risk;
Radioactive iodine;
Whole body scan;
Neck Ultrasound.

ABSTRACT

A combination of diagnostic whole body scan (DxWBS), neck ultrasound, and serum stimulated thyroglobulin (stim-Tg) is now recommended for the initial follow-up in patients with intermediate-risk differentiated thyroid cancer (DTC). However, previous studies demonstrated the low additional value of DxWBS for the detection of persistent disease. This study aimed to determine the necessity of using DxWBS for the initial follow-up of these patients. This retrospective analytical study included 126 patients with intermediate-risk DTC (according to 2015 ATA guidelines) after total thyroidectomy and received the first ^{131}I treatment. All patients underwent stim-Tg, neck ultrasound, and DxWBS at about 6–12 months after ^{131}I treatment. Persistent disease was defined as uptake outside the thyroid bed from DxWBS, uptake within the thyroid bed from DxWBS, or an abnormal ultrasound finding with cytologically or pathologically proven persistent disease, positive for serum anti-thyroglobulin antibody (TgAb), or stim-Tg of 1 ng/mL or more. The percentage difference for detection of persistent disease when using only neck ultrasound with stim-Tg compared to a combination with DxWBS was calculated. We considered non-inferior when the percentage difference is below five. Of the 126 patients with intermediate-risk DTC, persistent diseases were detected in 85 patients and identified by DxWBS, neck ultrasound, and stim-Tg in 24, 14, and 77 patients, respectively. Combined neck ultrasound and stim-Tg could detect persistent disease in 78 patients. Although using only neck ultrasound and stim-Tg could detect 8.2% (95%CI: 1.2 to 15.3) of patients with persistent disease less than that compared with using combined all three modalities, all patients with only positive DxWBS showed thyroid remnants, not the true persistent disease. These findings indicated that DxWBS may not be necessary for initial follow-up in patients with intermediate-risk DTC.

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Introduction

Patients with differentiated thyroid cancer (DTC) generally exhibit a favorable prognosis. While incidence rates of DTC are rising, survival rates have remained stable. These raise concerns about more tailored management[1]. Total thyroidectomy followed by ^{131}I treatment and life-long hormonal treatment were considered standard treatment of almost all these patients. Subsequently, response to therapy was evaluated by combination of diagnostic whole body scan (DxWBS), neck ultrasound (neck US), and serum stimulated thyroglobulin (stim-Tg) level at the initial follow-up (6-12 months after the first ^{131}I treatment). According to the latest American Thyroid Associated (ATA) guidelines, the 2015 ATA initial risk stratification system predicts risk of disease recurrence and guides follow-up management decisions. High-risk patients are recommended to get higher levels of thyrotropin (TSH) suppression and need more frequent follow-up visits than those who have lower risk of recurrence. While, subsequent DxWBS is not recommended for initial follow-up in patients with low risk who are clinically free of residual disease due to its low sensitivity in these patients, it is still recommended in patients with high-risk and intermediate-risk DTC[2].

Conversely, several previous studies demonstrated low value of DxWBS for detection of persistent disease in patients with intermediate-risk DTC[3-8]. However, there are no clear studies that demonstrate additional value of DxWBS in this patient group. In addition, some studies show that DTC patients with high initial stim-Tg, high stim-Tg, T3 or T4 stage, or a large tumor size are associated with a treatment failure founded by DxWBS[9-11] but there is no evidence that demonstrates the factors associated with the positive DxWBS results in patients with intermediate-risk DTC. These factors may be valuable for selection of patients who may benefit from DxWBS. Thus, this study primarily aimed to determine the necessity of using DxWBS for initial follow-up of patients with intermediate-risk DTC and secondarily aimed to determine the factors associated with the positive lesion from DxWBS in these patients.

Materials and methods

Study design

This retrospective cohort study collected record data from patients with DTC who previously received the first ^{131}I treatment at our tertiary care hospital between January 2015 and August 2017. After surgery, patients were evaluated for pathology results. The American Joint Committee on Cancer (AJCC) tumor-node-metastasis (TNM) staging, and 2015 ATA risk of recurrence system were used for patient's risk assessment. The patients were assigned to withdrawal thyroid hormone for four weeks and take a low iodine diet for two weeks. Serum TSH, free triiodothyronine (FT3), thyroglobulin (Tg), and anti-thyroglobulin antibody (TgAb) levels were measured within two days before the first ^{131}I oral administration. The DxWBS, neck US, and serum stim-Tg were generally evaluated at 6-12 months after initial treatment for detection of persistent disease.

^{131}I whole body scintigraphy and biochemical markers measurement

The post-therapy whole body scan (RxWBS) was performed at 5-7 days after oral administration of 1,110-5,550 MBq of ^{131}I. The DxWBS was performed 2-3 days after oral administration of 37-185 MBq of ^{131}I. All ^{131}I whole body scan (WBS) was performed by Discovery NM/CT 670 SPECT/CT system (General Electric, NY, USA) with a high-energy general purpose (HEGP) collimator. A measurement of serum Tg level was used THYROGLOBULINE IRMA, Cisbio Bioassays, France. Serum levels of thyroglobulin antibody (TgAb) and thyrotropin (TSH) were assayed using TGAB ONE STEP kits manufactured by Cisbio Bioassays in France, and RIA-gnost® hTSH kits manufactured by the same company, respectively.

Participants

We enrolled patients who fulfilled all of the following criteria; (1) pathological confirmed DTC, (2) underwent total or near-total thyroidectomy with or without lymph node dissection, (3) received the first ^{131}I treatment with performed RxWBS, (4) and underwent neck US, DxWBS, and stim-Tg at about 6-12 months after ^{131}I treatment. Furthermore, patients were excluded, if they had one or more of the following conditions; (1) second primary cancer detected before or during treatment, (2) previously received other treatment modality (eg., radiation therapy), (3) have a gross residual tumor, (4) have pathological high-risk feature(s); follicular thyroid carcinoma (FTC) with extensive vascular invasion (> 4 foci of vascular invasion), pathological N1 with any metastatic lymph node ≥ 3 cm in largest dimension, or macroscopic invasion of tumor into perithyroidal soft tissue, (5) distant metastasis detected before or after ^{131}I treatment, (6) being categorized as a low-risk DTC patient after being evaluated by pathological report and RxWBS, (7) serum TSH level during DxWBS study below 30 IU/mL. The remaining patients would be classified as intermediate risk of recurrence by ATA except some patients with pathological N1 micrometastases that were also included in the final analyses. One hundred and twenty six patients were needed according to the calculation from sample size needed to compare paired proportions: McNemar's Z-test, 1-sided[12] for testing a significant difference proportion that was set as five percent. This study was conducted following the Declaration of Helsinki and approved by the Khon Kaen University Ethics Committee (Reference number: HE621405). Informed consent was waived.

Data collection and interpretation

Data from a total of 126 patients (type of operation, surgical history, dose of ^{131}I administration, pathological and cytological results, level of serum TSH, Tg, TgAb, and neck ultrasound results) were collected. All patients' pathological reports were re-evaluated and re-classified using the eight edition AJCC/ TNM staging, and 2015 ATA risk of recurrence system. Interpretation of DxWBS, neck US and serum tumor marker was evaluated and defined as the following;

DxWBS: Both DxWBS planar image and single-photon emission computed tomography/computed tomography (SPECT/CT) images (if any) were independently reviewed by two nuclear medicine physicians who were blinded to clinical information, pathological results, treatment history, and other imaging studies via Xeleris 3.0 software or PACS. In case of disagreement, images were re-evaluated by both nuclear medicine physicians to make a conclusion. A negative DxWBS was made when there was; (1) no abnormal radioiodine uptake at the thyroid bed or outside thyroid bed region on planar image, or (2) suspicious of radioiodine uptake outside thyroid bed on planar images with proved negative lesion on SPECT/CT images. A positive DxWBS was made when there was; (1) abnormal radioiodine uptake outside thyroid bed from planar image, (2) abnormal radioiodine uptake at thyroid bed (thyroid remnant only), (3) suspicious of radioiodine uptake outside thyroid bed on planar image with proved positive lesion on SPECT/CT images, or (4) lesion from SPECT/CT-images represented metastasis (eg., enlarged mediastinal or cervical lymph node, multiple sharply marginated pulmonary nodules distributed randomly throughout the lung, smooth thickening of interlobular septa, peribronchovascular interstitial surrounding vessels and bronchus, or abnormal osteolytic/blastic bone lesion) without abnormal radioiodine uptake outside the thyroid bed on planar and SPECT images. An equivocal DxWBS was made when there were un-interpreted results from suspicious radioiodine uptake outside thyroid bed on planar images with/without SPECT/CT images.

Neck US: The neck US results and further cytological/pathological report was evaluated and interpreted as negative neck US when there was; (1) no abnormal findings on the neck US

results, (2) abnormal findings on the neck US results without subsequent pathological/ cytological investigation, or (3) abnormal findings on the neck US results with further pathological/ cytological investigation showing negative for thyroid carcinoma. A positive neck US was made only when there was abnormal finding on the neck US results with subsequent pathological/ cytological investigation proving positive for thyroid carcinoma.

Biological marker: Serum Tg and TgAb levels were recorded and interpreted as negative biochemical markers when serum stim-Tg level less than 1 ng/mL and negative for serum TgAb. A positive for biochemical markers was made when there was; (1) serum stim-Tg level 1 ng/mL or more, or (2) positive for serum TgAb.

Statistical analysis

The percentage of persistent-disease detection were compared between three (DxWBS, biochemical markers, and neck US) and two (biochemical markers and neck US) modalities methods by using McNemar's test. A two-sided *p*-value of less than 0.05 and five percentage difference was considered significant. The factors that predict positive DxWBS results (T-stage, extrathyroidal extension, cell type, cervical lymph node metastasis, stim-Tg, and initial stim-Tg) were identified by using bivariate logistic regression analysis. Factors were included in the multiple logistic regression analysis if their bivariate logistic regression *p*-value was < 0.25 or previously known strongly predictive factor from the previous studies such as an initial stim-Tg level more than 10 ng/mL[9,10]. Cohen's Kappa was used for evaluation of inter-observer reliability comparing the DxWBS (planar image or SPECT/CT images) interpretative results from two independent nuclear medicine physicians[13]. The categorical data were reported as number and percentage. The continuous data were reported as mean ± standard deviation (SD) or median and interquartile range. All statistical analyses were performed using STATA 10.1 (StataCorp LP, College Station, TX, USA).

Results

Of the total 328 intermediate-risk DTC patients first treated with ^{131}I were initially recruited in this study. Two hundred seventy-nine of these patients met all inclusion criteria. One hundred fifty-three patients were excluded due to; (1) second primary cancer, (2) having a pathological high-risk feature, (3) distant metastasis detected before or after ^{131}I treatment, (4) categorized as low-risk DTC, or (5) serum TSH level during DxWBS study below 30 IU/mL and 126 were finally evaluated in this study. The patients' characteristics are shown in table 1. The median age at diagnosis was 46 years (range: 14–74 years). Most of the patients were females (n = 104, 82.5%). The most common histological subtype was papillary carcinoma (n = 122, 96.8%) followed by follicular carcinoma (n = 4, 3.2%). Most of the patient's intermediate-risk feature was cervical lymph node metastasis (n = 92, 73%) followed by papillary thyroid carcinoma with vascular invasion (n = 53, 42.1%).

Table 1 Patient characteristics (n=126)

Characteristics	Sample	%
Median age at diagnosis, (IQR) [min-max], (year)	46 (30 - 57) [14 - 74]	
Age group, year		
< 55 years	89	70.6
≥ 55 years	37	29.4
Gender		
Female	104	82.5
Male	22	17.5
Type of operation		
Total thyroidectomy	125	99.2
Near-total thyroidectomy	1	0.8
Cell type		
Papillary thyroid carcinoma	122	
Classical variant	97	77.0
Tall cell variant	2	1.6
Diffuse sclerosing variant	1	0.8
Follicular variant	16	12.7
Oncocytic variant	2	1.6
Solid/trabecular variant	3	2.4
Micropapillary variant	12	9.5
Macropapillary variant	8	6.4
Mixed follicular and papillary variant	1	0.8
Follicular thyroid carcinoma	4	
Classical variant	3	2.4
Hurthle cell variant	1	0.8
Intermediate-risk characteristics		
Minimal ETE	39	31.0
Aggressive histological variant	4	3.2
PTC with vascular invasion	53	42.1
Cervical lymph node metastasis	92	73.0
Pathological diagnosis	70	55.6
RAI avid foci at neck from RxWBS	39	31.0

Table 1 Patient characteristics (n=126) (Cont.)

Characteristics	Sample	%
TNM stage (AJCC/TNM 8thed.)		
T1N0	10	7.9
T1N1	41	32.5
T2N0	12	9.5
T2N1	34	27.0
T3N0	11	8.7
T3N1	18	14.3
Stage group		
I	99	78.6
II	27	21.4
T stage		
1a	21	16.7
1b	30	23.8
2	46	36.5
3a	29	23.0
N stage		
0	34	27.0
1	43	34.1
1a	24	19.1
1b	25	19.8
Median tumor size (IQR) [min-max], (cm)	2.65 (1.5 - 4) [0.1 - 8]	
Dose of ^{131}I treatment (mCi)		
30	11	8.7
100	4	3.2
150	111	88.1

Note: The TNM stage, which is based on the examination of the surgical specimen, provides information on the primary tumor (T) and lymph node (N) metastasis. A tumor measuring up to 1 cm confined to the thyroid is classified as stage T1a, while a tumor measuring more than 1 cm but up to 2 cm confined to the thyroid is classified as stage T1b. For tumors measuring more than 2 cm but up to 4 cm confined to the thyroid, they are classified as stage T2, and for tumors larger than 4 cm confined to the thyroid, they are classified as stage T3a. In terms of lymph node involvement, a stage N0 indicates no evidence of regional lymph node metastasis, while N1a indicates metastasis to cervical lymph nodes level VI or VII. Metastasis to cervical lymph nodes level I, II, III, IV, or V is classified as N1b.

Abbreviations: IQR, interquartile range; ETE, extrathyroidal extension; PTC, papillary thyroid carcinoma; RAI, radioactive iodine; RxWBS, post treatment whole body scan; TNM, tumor-node-metastasis; AJCC, the American Joint Committee on Cancer.

Detection of persistent disease

Of the total 126 intermediate-risk DTC patients, persistent disease was found in 14 patients by neck US, 77 patients by biochemical markers, and 24 patients by DxWBS. Among 24 patients with positive DxWBS results, one patient had cervical lymph node metastasis, two had cervical lymph node metastasis with bone metastasis, one had cervical lymph node metastasis with thyroid remnant, and 20 with thyroid remnant only. Furthermore, four patients had equivocal DxWBS results, including one with suspicion of mediastinal lymph node metastasis, two with suspicion of cervical lymph node metastasis, and one with suspicion of pulmonary metastasis. Two of these patients also had serum stim-Tg levels more than 1 ng/mL but the rest showed only equivocal DxWBS results as shown in table 2. Therefore, the overall number of patients with persistent disease detected by neck US, biochemical markers, or DxWBS was 85.

Table 2 Overall persistent disease detection (n=87)

Test	Total	
	Number of patients (%)	95 % CI
Biochemical markers	77 (88.5)	79.88; 94.35
Positive for serum Tg only	57 (65.5)	54.56; 75.39
Positive for serum TgAb only	14 (16.1)	9.09; 25.52
Positive for serum Tg and TgAb	6 (6.9)	2.57; 14.41
Neck ultrasound	14 (16.1)	9.09; 25.52
Diagnostic whole body scan (DxWBS)	24 (27.6)	18.54; 38.21
Positive sites:		
Functioning cervical lymph node metastasis	4 (4.6)	1.27; 11.36
Cervical lymph node metastasis only	1 (1.2)	0.03; 6.24
Cervical lymph node metastasis with bone metastasis (SPECT/CT)	1 (1.2)	0.03; 6.24
Cervical lymph node metastasis with functioning bone metastasis	1 (1.2)	0.03; 6.24
Cervical lymph node metastasis with thyroid remnant	1 (1.2)	0.03; 6.24
Thyroid remnant	20 (23.0)	14.64; 33.25
Equivocal	4 (4.6)	1.27; 11.36
Suspicious for lymph node metastasis	3 (3.5)	0.72; 9.75
Suspicious for mediastinal lymph node metastasis	1 (1.2)	0.03; 6.24
Suspicious for cervical lymph node metastasis	2 (2.3)	0.28; 8.06
Suspicious for pulmonary metastasis	1 (1.2)	0.03; 6.24

Note: The total number of 87 patients is derived from 85 patients who had persistent disease detected by neck ultrasound, biochemical markers, or DxWBS, with an additional two patients with equivocal DxWBS only.

Abbreviations: Tg, thyroglobulin; TgAb, anti-thyroglobulin antibodies; SPECT/CT, single photon emission computed tomography/computed tomography.

The percentage difference for detection of persistent disease

Patients with equivocal DxWBS results may or may not actually have the persistent disease. Therefore, we decided to analyze the results in both ways, treating equivocal DxWBS results as positive and as negative.

When patients with equivocal DxWBS were grouped as positive DxWBS

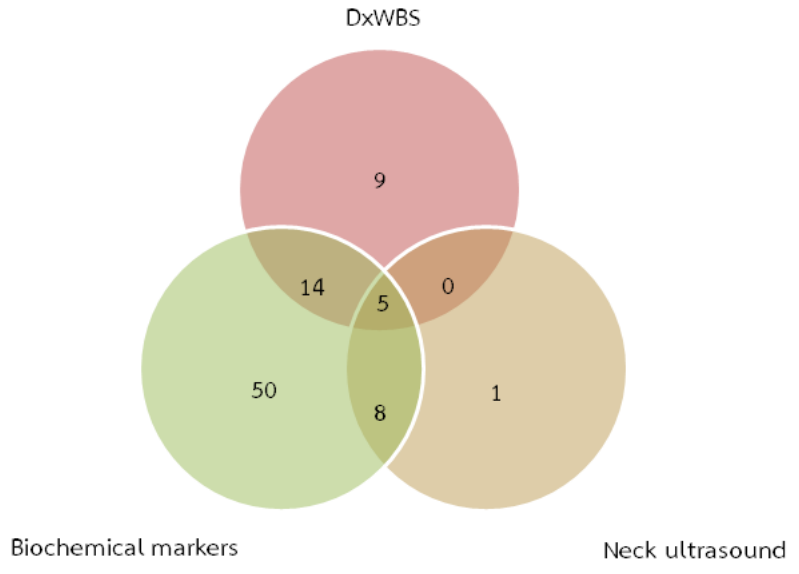
Persistent disease was found as follows; only from biochemical markers in 50 patients, neck US in one patient, or DxWBS in nine patients (seven patients with thyroid remnant and two patients with equivocal DxWBS); both neck US and biochemical markers in eight patients; both biochemical markers and DxWBS (one patient with cervical lymph node metastasis, one patient with cervical lymph node and bone metastasis, one patient with cervical lymph node metastasis and thyroid remnant, eight patients with thyroid remnant only, two patients with equivocal DxWBS, and one patient with thyroid remnant and equivocal DxWBS) in 14 patients; combined neck US, biochemical markers, and DxWBS (four patients with thyroid remnant, one patient with cervical lymph node and bone metastasis) in five patients as shown in table 3 and figure 1 (A). Therefore, a total number of patients with persistent disease detected from neck US, biochemical markers, or DxWBS were 87. Persistent disease identified by both biochemical markers and neck US in 78 patients. The percentage difference for detection of persistent disease when using only in the neck US with biochemical markers compared to combination with DxWBS was 10.3% (95% CI 2.8-17.7, *p*-value = 0.004) as shown in table 4.

Table 3 Persistent disease detection (utilizing dual analysis: initially classifying equivocal DxWBS findings as positive and subsequently as negative)

	Positive-DxWBS group (n = 87)		**Negative-DxWBS group (n = 85)**	
	Number of patients (%)	**95%CI**	**Number of patients (%)**	**95%CI**
Biochemical markers only	50 (57.5)	46.41; 68.01	52 (61.2)	49.99; 71.56
Neck ultrasound only	1 (1.2)	0.03; 6.24	1 (1.2)	0.03; 6.38
Diagnostic whole body scan only	9 (10.3)	4.84; 18.73	7 (8.2)	3.38; 16.23
Biochemical markers or neck ultrasound	78 (89.7)	81.27; 95.16	78 (91.8)	83.77; 96.62
Biochemical markers or diagnostic whole body scan	86 (98.9)	93.76; 99.97	84 (98.8)	93.62; 99.97
Neck ultrasound or diagnostic whole body scan	37 (42.5)	31.99; 53.59	33 (38.8)	28.44; 50.01
Biochemical marker or diagnostic whole body scan or neck ultrasound	87 (100.0)	95.85; 100	85 (100.0)	95.75; 100

Abbreviations: DxWBS, diagnostic whole body scan.

(A) Grouped as positive DxWBS



(B) Grouped as negative DxWBS

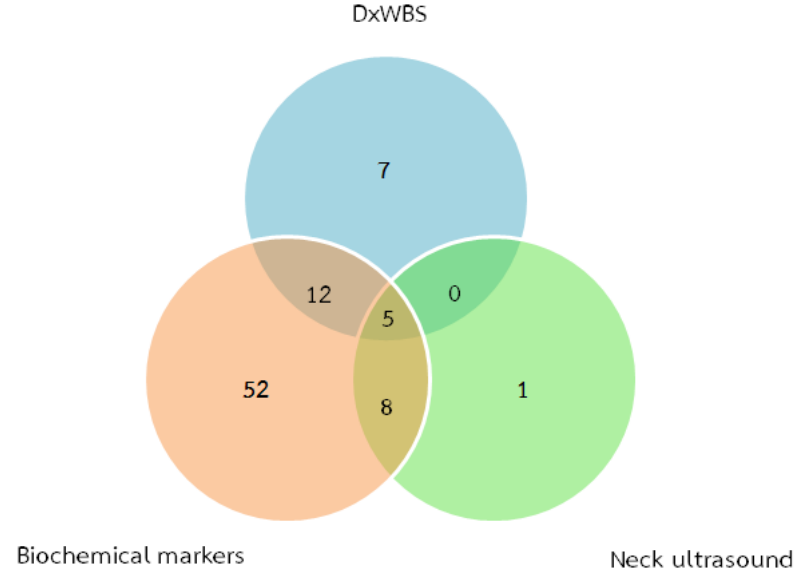


Figure 1 The number of patients with persistent disease detected by DxWBS, neck US, or biochemical markers.

Table 4 Percentage difference for detection of persistent disease when using only in the neck ultrasound with stim-Tg compared to combination with DxWBS in all patients (utilizing dual analysis: initially classifying equivocal DxWBS findings as positive and subsequently as negative)

Biochemical marker and diagnostic whole body scan and neck ultrasound					
		Positive-DxWBS group Number (%)		Negative-DxWBS group Number (%)	
Biochemical markers and neck ultrasound		Positive	Negative	Positive	Negative
	Positive	78 (89.7)	-	78 (91.8)	-
	Negative	9 (10.3)	-	7 (8.2)	-
	p-value	0.004		0.016	
Proportion difference (95%CI)		10.3% (2.8; 17.7)		8.2% (1.2 to 15.3)	

Note: Proportion of detection of persistent disease were compared between three methods (DxWBS, biochemical markers, neck US) and two methods (biochemical markers and neck US) using McNemar's test. Significance was determined by a two-sided *p*-value below 0.05 and a five percentage point difference.
Abbreviations: stim-Tg, serum stimulated thyroglobulin; DxWBS, diagnostic whole body scan.

When patients with equivocal DxWBS were grouped as negative DxWBS

Persistent disease was found as follows; only from biochemical markers in 52 patients, neck US in one patient, or DxWBS in seven patients (thyroid remnant for all); both neck US and biochemical markers in eight patients; both biochemical markers and DxWBS (one patient with cervical lymph node metastasis, one patient with cervical lymph node and bone metastasis, one patient with cervical lymph node metastasis and thyroid remnant, and nine patients with thyroid remnant only) in 12 patients; combined neck US, biochemical markers, and DxWBS (four patients with thyroid remnant, one patient with cervical lymph node and bone metastasis) in five patients as shown in table 3 and figure 1 (B).

As grouped patients with equivocal DxWBS within the negative DxWBS group, the total number of patients with persistent disease detected from neck US, biochemical markers, or DxWBS was 85. Persistent disease identified by both biochemical markers and neck US in 78 patients. The percentage difference for detection of persistent disease when using only in the neck US with biochemical markers compared to combination with DxWBS was 8.2% (95% CI 1.2-15.3, *p*-value = 0.016) as shown in table 4.

Factors associating with positive DxWBS

Out of 126 intermediate-risk DTC patients, 28 had abnormal DxWBS results; 24 were positive, and four were equivocal. Bivariate logistic regression analysis found no significant factors associated with positive DxWBS, even when equivocal results were considered positive or negative. Previous strong predictive factors were analyzed using multiple logistic regression, which also showed no significance. However, the initial stim-Tg level more than 10 ng/mL tended to have an association with positive DxWBS in both analyses (*p*-value = 0.062, adjusted OR = 3.77 and *p*-value = 0.087, adjusted OR = 3.30).

Inter-observer reliability

One hundred twenty-eight lesions on planar images of DxWBS were detected from 126 patients by two readers. There were four lesions in two patients that had non-concordant results. The first patient was interpreted to have two lesions by reader A but interpreted to have one lesion by reader B. Conversely, the second patient was interpreted to have two lesions by reader B but interpreted to have one lesion by reader A. Other two lesions in one patient were interpreted concordantly by both readers. Consequently, the compared lesions from the planar images of

DxWBS were 127. A strong overall agreement (114 lesions, 89.76%) was found between two readers, with a Kappa score of 0.75 which was considered substantial[13]. Of the total 29 patients who did the SPECT/CT images, two lesions in one patient were concordantly interpreted by both readers. Thus, overall, 31 lesions from SPECT/CT images were compared. From the SPECT/CT images interpretation, there was also strong agreement (27 lesions, 87.1%) between two readers, with Kappa score of 0.77 which was considered substantial[13].

Discussion

The findings of this retrospective study show that the percentage difference for detection of persistent disease when using only in the neck ultrasound with biochemical markers compared to combination with DxWBS was 10.3% when patients with equivocal DxWBS were grouped as positive DxWBS and was 8.2% when they were grouped as negative DxWBS (Table 4). Even when patients with equivocal-DxWBS grouping as positive DxWBS or negative DxWBS, the percentage difference is more than five percent which is considered significant. However, a follow-up evaluation of equivocal DxWBS patients at the median follow-up time of three years showed no further management needed in all these patients. We thus assumed that the equivocal-DxWBS patients had no true persistent disease and the percentage of difference from this study was only 8.2%.

Previously, there was no published literature studying the percentage difference for detection of persistent disease when using only neck US with biochemical markers compared to combination with DxWBS in patients with intermediate-risk DTC. However, the percentage difference can be calculated from individual data in some previously studied sources [3-5,7]. A percentage difference calculated from a study that determined the necessity of a DxWBS after ^{131}I ablation in intermediate-risk DTC patients by Eon et al[7] was 7.8%. This minimally lower value of a percentage difference compared with our study may occur from that the neck US was not used for evaluation of persistent disease in this study. Moreover, only intermediate-risk-DTC patients who received 1,110 MBq of ^{131}I ablation even when more than half of them were stage III (63.1%) and no patient with metastasis from RxWBS were included in this study. This may represent that they selected only patients with low-risk features. This may be another reason for the lower percentage difference. Similarly to a study by Cailleux et al[3] which calculated the percentage difference as 5.9%. This study assessed whether routine control DxWBS should be routinely performed within 1 year after 3,700 MBq of ^{131}I treatment in low-to-high risk DTC patients. The conclusion that DxWBS only confirmed the completeness of thyroid ablation was made by only thyroid remnants was detected from DxWBS in 20 patients. Even though they included all-risk recurrence patients, there were only patients who had no functioning uptake outside the thyroid bed from RxWBS; this may cause a lower percentage difference.

Some studies showed higher percentage differences compared to our study. Pacini et al[4] evaluated the diagnostic accuracy of recombinant human TSH (rhTSH)-stimulated WBS and serum Tg alone or in combination in DTC patients. The percentage difference of this study was calculated as 15.69%. This may be explained by they included some 2015 ATA high-risk DTC patients; however, the characteristic of patients was not sufficiently provided. Rosario et al[5] determined whether DxWBS with stim-Tg was necessary for patients with negative RxWBS, neck US, suppressed thyroglobulin, and TgAb after ablation, who are considered to be at high or intermediate risk for recurrence (only patients with large tumor size or tumor extension beyond thyroid capsule). At the follow-up time of 8-12 months after radioiodine ablation, they found that there was no functioning metastasis from DxWBS in all patients, only thyroid remnant was detected in 46 patients from a total 318 patients. Furthermore, when dividing patients

into two groups by stim-Tg level (below 1 ng/mL or at least 1 ng/mL), thyroid remnant was detected in 33 patients from stim-Tg below 1 ng/ mL group and in 13 patients from stim-Tg at least 1 ng/mL group. At the median follow-up time of 60 months, a recurrent disease occurred in only one patient from stim-Tg below 1 ng/ mL grouped. They concluded that DxWBS can be avoided in patients who have the same included characteristics in their study. The calculated percentage difference from this study was 10.4% which is slightly higher than our study. Therefore, the percentage difference from our study cannot completely be compared to others because of many differences in study setting. These included; (1) each study have different criteria for persistent disease; some studies included only patients with negative neck US results[5]; or some studies did not use neck US results[3,7], (2) some studies included patients with other risks of recurrence[3-5] or included only intermediate-risk DTC patients with low-risk features[7].

Despite having a significant percentage difference, all positive-DxWBS lesions in our study were only thyroid remnants. This is consistent with findings from several previous studies[3,5,7] including study by Kim et al[14] which evaluated the clinical outcomes of DTC patients with thyroid remnants detected by DxWBS after initial therapy. After follow-up DTC patients whose has none of the following; (1) functioning metastasis, (2) serum TgAb more than 100 U/mL, and (3) serum TSH at the time of DxWBS performing lower than 30 IU/mL, recurrent disease occurred in 72 patients dividing into 5 patients (20%) from the remnant-positive group (total 25) and 67 patients (12%) from the remnant-negative group (total 547). There was no significant difference in disease-free survival (DFS) between these two groups at the median follow-up time of 65.7 months. They concluded that DxWBS was not needed in these patients. Interestingly, 12 patients from a total 25 patients in the remnant-positive group had negative for neck US and undetectable stim-Tg which has the same characteristic as patients with positive DxWBS in our study. After the end of study, none of them showed recurrent disease. Assuming from this finding, if we follow-up our patients with positive DxWBS, they may also have no recurrent disease.

Although we found no significant factor associated with positive DxWBS results, initial stim-Tg at least 10 ng/mL tended to have an association with positive DxWBS. This finding is similar to those in the previous studies which found that the initial stim-Tg at least 10 ng/mL had an association with positive DxWBS results or stim-Tg more than 2 ng/mL[9,10]. One of the causes of unidentified significant factor associated with positive DxWBS from our study may be from the low number of positive DxWBS patients in our study. Further prospective studies with a larger number of patients are required to confirm if the initial stim-Tg of at least 10 ng/mL is associated with positive DxWBS results in these patients.

This study has limitations. Uncontrolled data were recorded from a retrospective study. Intermediate-risk DTC patients were not identically defined as ATA 2015 criteria. Patients with one or more cervical lymph node metastases smaller than 3 cm were included. The follow-up time of this study was only three years, which may not be sufficient to detect recurrence[15]. Further study should employ a prospective design, with stringent criteria for patient selection, and extended period of follow-up.

Conclusion

DxWBS add little value for initial follow up in intermediate-risk DTC patients who had done neck US and stim-Tg. This may not be necessary in patients with intermediate-risk DTC.

Take home messages

Combined neck US and stim-Tg might be a sufficient tool for identifying persistent disease during the initial post-treatment monitoring of patients with intermediate-risk DTC.

Conflicts of interest

The authors declare no conflict of interest.

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Cytogenetic Abnormalities in Hematolymphoid Tumors: Insight from Karyotyping of Bone Marrow Specimens in Northeast Thailand

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KEYWORDS
Cytogenetic abnormalities; Hematolymphoid tumors; Karyotyping; Bone marrow specimens.

ABSTRACT

Hematolymphoid tumors are classified into 1) lymphoid and 2) myeloid and histiocytic/dendritic neoplasms and are commonly caused by chromosomal abnormalities, such as chromosomal translocations. Previous studies reveal that genetic landscapes in acute myeloid leukemia (AML) vary between national populations. Therefore, this study aims to investigate the frequency of cytogenetic abnormalities using the conventional karyotype technique among hematolymphoid tumor patients in Northeast Thailand. Our finding of 314 hematolymphoid tumor patients demonstrates that the most common finding is chronic myeloid leukemia (CML) (187 cases, 59.55%), while AML was observed in 53 cases (16.88%). The third most prevalent finding is acute lymphoblastic leukemia (ALL), comprising 26 cases (8.28%). Other findings of hematolymphoid tumor patients in this region reveal myelodysplastic neoplasms (MDS) (19 cases, 6.05%) and lymphoma (9 cases, 2.87%). Among the 314 cases examined, the result of conventional karyotype shows most cases are normal chromosome (249 cases, 79.30%), while chromosome abnormality was seen in 26 cases (8.28%). Unfortunately, no metaphase or unsuccessful karyotype was revealed in 39 cases (12.42%). The most prevalent abnormality of 26 cases is the translocation between chromosomes 9q34 and 22q11.2, observed in 20.93% of cases and prominently associated with CML. Moreover, chromosome Y loss is demonstrated in both CML and AML (4 cases, 9.30%). Other chromosome aberrations are revealed in this study, including monosomy 21, marker chromosome (s), monosomy X, and trisomy 8. In conclusion, this study suggests that CML is the most common hematolymphoid tumor in the northeast Thai population, frequently associated with the translocation of chromosomes 9q34 and 22q11.2. This finding contributes to our knowledge about hematolymphoid tumors in specific regions in Thailand which might be useful for management system of hematolymphoid tumor diagnosis and treatment.

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Introduction

Hematolymphoid tumors originate from hematopoietic and lymphoid tissues, affecting the blood, bone marrow, lymph nodes, and the lymphatic system[1]. Consequently, these tumors can cause aplasia, myeloproliferative, and lymphoproliferation, including leukemias and lymphomas[2,3]. Unlike solid tumors, a common cause of these diseases is chromosomal abnormalities, such as chromosomal translocations[4,5]. Two groups of hematolymphoid tumors are classified by the 5th edition of the WHO classification as lymphoid and myeloid and histiocytic/dendritic neoplasms. Lymphoid neoplasms are categorized into three subgroups: B-cell lymphoid proliferation and lymphomas such as acute lymphoblastic leukemia (ALL) and chronic lymphocytic leukemia (CLL), T-cell and NK-cell lymphoid proliferations and lymphomas, and stroma-derived neoplasms of lymphoid tissues. Similarly, myeloid and histiocytic/dendritic neoplasms are classified into nine subgroups as follows; (A) myeloproliferative neoplasms (MPN) including chronic myeloid leukemia (CML), polycythemia vera (PV), essential thrombocythemia, primary myelofibrosis, (B) myelodysplastic neoplasms (MDS), (C) myelodysplastic/myeloproliferative neoplasms (MDS/MPN) such as chronic myelomonocytic leukemia (CMML), (D) acute myeloid leukemia (AML), (E) secondary myeloid neoplasms, (F) myeloid/lymphoid neoplasms with eosinophilia and tyrosine kinase gene fusions (MLN-TK), (G) acute leukemias of mixed or ambiguous lineage, (H) histiocytic/dendritic cell neoplasms, (I) genetic tumor syndromes with predisposition to myeloid neoplasia[6,7].

A different approach to diagnosing and treating hematolymphoid tumors is typically used. Diagnosis is usually based on complete blood counts, bone marrow examination, and observation of symptoms[8]. Bone marrow biopsy should confirm the diagnosis and classification[6-9]. These tests are essential and must be conducted regularly to provide a prognosis that will influence the patient's choice of appropriate treatment. Additionally, cytogenetic testing is crucial for diagnosis, prognosis, and post-treatment monitoring[6-8]. Deletion or duplication, which may occur in part or the entire chromosome, has been implicated in developing many diseases.

Over the past 60 years, cytogenetic analysis of hematologic malignancies has significantly advanced, employing chromosome studies and molecular techniques to identify disease-specific abnormalities. With over 600 fusion genes and 1,000 balanced translocations discovered, these methods have become pivotal in diagnosing, treating, and prognosing hematologic cancers[10]. The molecular cytogenetic phenotype has led to tailored treatments, exemplified by successful gene-targeted therapy in CML[11]. There are many cytogenetic techniques, one of the most commonly used being the conventional karyotype[12,13]. This method can distinguish chromosome abnormalities by examining the number and structure. Cytogenetic abnormalities play a crucial role in the pathogenesis, providing more details on these disorders' prognosis, diagnosis, and clinical outcomes [6,7].

AML patients in Seattle, USA, with unsuccessful cytogenetics (UC) had a lower response to chemotherapy treatment and were associated with a poor prognosis[14]. In China, AML patients with cytogenetic abnormalities have shorter overall survival than patients with normal karyotypes[15]. In Thailand, 44.1% of MDS patients have chromosome abnormalities, with the most common abnormalities being monosomy 7 and trisomy 8, each detected in 26.7% of the patients. Furthermore, the age of Thai MDS patients is lower than that of the Western population. Moreover, the genetic landscapes of AML patients vary between national populations[16]. Recently, the report of chromosomal abnormalities in MDS patients in upper Northern Thailand showed that the frequency and pattern differed from other populations. In addition, the percentage of blasts in bone marrow in MDS patients with abnormalities of chromosome

7 and other complex chromosomes has a higher risk of progressing to AML[17]. With its unique demographic and environmental factors, Northeast Thai population may exhibit distinctive cytogenetic abnormalities in hematologic malignancies. Therefore, this research seeks to fill the existing knowledge gap by investigating the spectrum and frequency of cytogenetic abnormalities among hematolymphoid tumor patients in this region of Thailand.

Materials and methods

Study design and participants

A retrospective analysis of the karyotype results was conducted on bone marrow specimens obtained from patients sent to the Cytogenetic Laboratory, Department of Pathology, Srinagarind Hospital, Faculty of Medicine, Khon Kaen University, in 2020-2021. The standard of Cytogenetic Laboratory has been accredited by The Royal of Pathologists of Thailand. The specimens underwent conventional karyotyping techniques to detect chromosomal abnormalities. A total of 580 cases, comprising 298 cases in 2020 and 282 cases in 2021, were included in this study. The exclusion criteria for this study included 1) cases not diagnosed with hematolymphoid tumors or suspicious diagnoses and 2) cases with incomplete clinical data.

Demographic data, including age and gender, and clinical data, including the pathologist and internist diagnosis, were correlated with cytogenetic findings. This study performed descriptive statistical analyses. The Institutional Review Board of Khon Kaen University approved the ethical considerations for this study (HE661237).

Conventional karyotyping

The conventional karyotype analysis protocol outlined in this study involved a comprehensive process for cell culture, harvest, spread slide preparation, staining with Trypsin and Giemsa, and subsequent interpretation, as shown in the supplement data. Briefly, cells were initially cultured with RPMI1640 and fetal bovine serum for 24 hours. Methotrexate treatment was then introduced, followed by centrifugation, supernatant removal, and the addition of RPMI 1640. Thymidine treatment and subsequent incubation complete the cell culture process.

Harvesting involved colcemid treatment, potassium chloride (KCl) incubation, and fixation with methanol: acetic acid fixative. The cells underwent multiple centrifugation and fixative steps before being stored at 4 °C. The spread slide protocol included soaking cleaned slides in distilled water, gently resuspending harvested cells, applying samples to slides, drying on a heated platform, and labeling. Trypsin and Giemsa staining was followed in four Coplin jars, with trypsin treatment, phosphate buffer rinse, Giemsa dyeing, and dilute water washes.

The stained slides were then analyzed using light microscopy under 10X and 100X magnification. Two independent cytogeneticists conducted the analysis. Metaphase cell numbers were determined at 100X magnification and analyzed using Ikaros Karyotyping Software (MetaSystems, Altlussheim, Germany) and GenASIs Bandview software (Applied Spectral Imaging, California, USA). Interpretation using the International System for Human Cytogenomic Nomenclature System 2020 (ISCN 2020) was employed for result interpretation[18]

Results

The prevalence of hematolymphoid tumors in Srinagarind Hospital, Northeast Thailand

This study encompassed 580 cases, with stringent exclusion criteria to ensure a focused investigation. Cases lacking diagnoses of hematolymphoid tumors or presenting with suspicious diagnoses and those with incomplete clinical data were excluded from the analysis. Following these criteria, a robust dataset of 314 cases with confirmed hematolymphoid tumors emerged (Table 1). The gender distribution within

this subset revealed 153 cases (48.70%) among females aged 0 to 81 years. Males constituted 161 cases (51.30%), ranging from 2 to 89 years.

The five most prevalent diseases were identified among the hematolymphoid tumors investigated in this study (Table 1). CML emerged as the most common, constituting a substantial portion with 187 cases (59.55%). Following closely, AML was observed in 53 cases (16.88%) of the study cohort. ALL was the third most prevalent, comprising 26 cases (8.28%). MDS were identified in 19 cases 6.05%), and lymphoma cases totaled 9 (2.87%).

Table 1 The demographic data of 314 hematolymphoid tumor cases

Demographic data	Number of Cases (%)
Sex, cases (%)	
Male	161 (51.30%)
Female	153 (48.70%)
Median Age, years (range)	
0-50 years	159 (50.64%)
51-89 years	155 (49.36%)
Diagnosis, cases (%)	
- CML	187 (59.55%)
- AML	53 (16.88%)
- ALL	26 (8.28%)
- MDS	19 (6.05%)
- Lymphoma	9 (2.87%)
- Other*	20 (6.37%)
Total	314

Note: * Others, including ET for 6 cases, PCM for 5 cases, PV for 5 cases, PMF for 2 cases, CLL for 1 case, and CMML for 1 case.

Abbreviations: CML, chronic myeloid leukemia; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; MDS, myelodysplastic neoplasms; ET, essential thrombocythemia; PCM, plasma cell (multiple) myeloma; PV, polycythemia vera; PMF, primary myelofibrosis; CLL, chronic lymphocytic leukemia; CMML, chronic myelomonocytic leukemia.

The prevalence of normal karyotype, no metaphase cells, and cytogenetic abnormalities in hematolymphoid tumors

The karyotype analysis yielded results across three distinct categories in the studied cohort. Among the 314 cases examined, 26 cases (8.28%) displayed abnormal karyotypes (cytogenetic abnormalities), with diagnoses further categorized into specific hematolymphoid disorders (Tables 2 and 3). Utilizing the International System for Human Cytogenomic Nomenclature 2020 (ISCN 2020) for accurate interpretation, the breakdown of abnormal karyotypes included 11 cases of CML, 9 cases of AML, 2 cases of ALL, 2 cases of lymphoma, and 1 case of PV and 1 case of primary myelofibrosis (PMF) (Table 2). Additionally, 39 cases (12.42%) exhibited no metaphase cells, and the majority, 249 cases (79.30%), displayed a normal karyotype.

Table 2 The prevalence of normal karyotype, no metaphase cells, and cytogenetic abnormalities in hematolymphoid tumors

Hematolymphoid tumors	Karyotype, cases (%)			Total cases (%)
	Normal chromosome	No metaphase cell	Abnormal chromosome	
CML	157 (83.96%)	19 (10.16%)	11 (5.88%)	187 (100%)
AML	33 (62.26%)	11 (20.75%)	9 (16.98%)	53 (100%)
ALL	19 (73.08%)	5 (19.23%)	2 (7.69%)	26 (100%)
MDS	17 (89.47%)	2 (10.53%)	0 (0.00%)	19 (100%)
Lymphoma	6 (66.67%)	1 (11.11%)	2 (22.22%)	9 (100%)
*Other	17 (85.00%)	1 (5.00%)	2 (10.00%)	20 (100%)
Total	249 (79.30%)	39 (12.42%)	26 (8.28%)	314 (100%)

Note: * Others, including ET for 6 cases, PCM for 5 cases, PV for 5 cases, PMF for 2 cases, CLL for 1 case, and CMML for 1 case.

Abbreviations: CML, chronic myeloid leukemia; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; MDS, myelodysplastic neoplasms; ET, essential thrombocythemia; PCM, plasma cell (multiple) myeloma; PV, polycythemia vera; PMF, primary myelofibrosis; CLL, chronic lymphocytic leukemia; CMML, chronic myelomonocytic leukemia.

Table 3 The 26 cases of abnormal karyotyping were divided into diagnostic groups. The karyotype interpretation used the International System for Human Cytogenomic Nomenclature 2020 (ISCN 2020)

Diagnostic group	Karyotype (26 cases)	Sex	Age (year)
CML (11 cases)	46,XY,t(9;22)(q34;q11.2)[20]/46,XY[4]	Male	38
	46,XY,t(9;22)(q34;q11.2)[6]	Male	43
	46,XY,t(9;22)(q34;q11.2)[15]/46,XY[60]	Male	43
	46,XY,t(9;22)(q34;q11.2)[12]	Male	32
	46,XY,t(9;22)(q34;q11.2)[25]	Male	43
	46,XY,t(9;22)(q34;q11.2)[25]	Male	42
	46,XX,t(9;22)(q34;q11.2)[25]	Female	48
	46,XY,t(9;22)(q34;q11.2)[3]/46,XY[22]	Male	44
	46,XY,t(9;22)(q34;q11.2)[42]/46,XY[25]	Male	58
	45,X,-Y[9]/46,XY[17]	Male	52
	47,XY,+15[3]/46,X,-Y,+15[8]/46,XY[22]	Male	61

Table 3 The 26 cases of abnormal karyotyping were divided into diagnostic groups. The karyotype interpretation used the International System for Human Cytogenomic Nomenclature 2020 (ISCN 2020) (Cont.)

Diagnostic group	Karyotype (26 cases)	Sex	Age (year)
AML (9 cases)	46,X,-X,+mar[25]	Female	44
	47,XX,add(1)(p36.1),-6,-8,+mar1,+mar2,+mar3[5]	Female	0
	47,XX,+21[15]/46,XX[3]	Female	60
	48,XY,+8,+11[2]/46,XY[7]	Male	77
	46,XX,del(8)(q22),-21,+mar[25]	Female	12
	46,XX,add(15)(q26),-16,-21,+mar1,mar2[42]/46,XX[1]	Female	16
	47,XY,+4[11]/46,XY[28]	Male	66
	47,XX,+8[8]	Female	20
	45,X,-Y,t(8;21)(q22;q22.3)[10]	Male	5
ALL (2 cases)	46,XY,add(11)(q25)[25]	Male	14
	47,XX,+22[3]	Female	22
Lymphoma (2 cases)	51-68<3n>,XXX,+X,+1,+6,+18,+19,+19,+21,[17]	Female	69
	46,X,-X,-10,+mar1,+mar2[1]/46,X,-X,+18[1]/ 48,XX,del(1)(q25),+14,-18,-18,+mar1,+mar2,+mar3[1]	Female	40
PV (1 case)	46,XY,add(15)(q10)[15]	Male	58
PMF (1 case)	46,XX,t(11;12)(p15;q15)[11]	Female	68

Note: The number in [ ] represents the metaphase cell count for the karyotype. The "/" represents different clones of metaphase cells, also called mosaicism.

Abbreviations: p, short arm of chromosome; q, long arm of chromosome; t, translocation; add, additional material of unknown origin; del, deletion; mar, marker chromosome; 3n, near-triploidy; CML, chronic myeloid leukemia; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; PV, polycythemia vera; PMF, primary myelofibrosis.

Recurrent rates of cytogenetic abnormality

The research findings on cytogenetic abnormalities in hematolymphoid tumors, encompassing 43 cases, reveal a diverse spectrum of recurrent patterns (Table 4). The most prevalent abnormality is the Philadelphia chromosome, resulting from the translocation between chromosomes 9q34 and 22q11.2, observed in 20.93% of cases and prominently associated with CML. For example, the karyotype of the 32-year-old male diagnosed with CML reveals 46,XY,t(9;22)(q34;q11.2)[12] as shown in figure 1A. In addition, marker chromosomes are identified in 9.30% of cases, indicating associations with AML and lymphoma. A karyotype of the 16-year-old female diagnosed with AML reveals 46,XX,add(15)(q26),-16,-21,+mar1,+mar2[42]/46,XX[1] indicating that 42 metaphase cells with additional chromosome material attached to 15q26, monosomy 16, monosomy 21, and 2 different marker chromosomes and 1 metaphase cell with normal female chromosome as figure 1B. Notable occurrences include the loss of the Y chromosome in males (6.98%), linked to CML and AML, and monosomy 21 (Figure 1B) and trisomy 8 (4.65% each) associated with AML (Figure 1F and 1G). Monosomy X in

females (4.65%) is also identified in AML and lymphoma cases (Figure 1C-1F). The mosaicism chromosome of the lymphoma patient with ISCN: 46,X,-10,+mar1,+mar2[1]/46,X,+18[1]/48,XX, del(1)(q25),+14,-18,-18,+mar1,+mar2,+mar3[1] was seen in 3 metaphase cells as figure 1C, 1D, 1E, respectively. Figure 1C demonstrates monosomy 10, monosomy X, and 2 different marker chromosomes (ISCN: 46,X,-10,+mar1,+mar2[1]). Additionally, the metaphase cell of Figure 1D indicates monosomy X and trisomy 18 (ISCN: 46,X,+18[1]). The metaphase cell of Figure 1E demonstrates deletion of 1q25, trisomy 14, nullisomy 18, and 3 marker chromosomes (ISCN: 48,XX,del(1)(q25),+14,-18,-18,+mar1, +mar2, +mar3[1]). The example of the 77-year-old male diagnosed with AML reveals a result of 48, XY,+8,+11[2]/46,XY[7] in 2 metaphase cells, while others are normal male chromosomes. This abnormality indicates trisomy 8 and trisomy 11 in 2 metaphase cells, as shown in figure 1F. The last sample with a representative of an abnormal karyotype is the 20-year-old female diagnosed with AML and reveals a result of 47,XX,+8[8], indicating trisomy 8 in 8 metaphase cells. Various other rare abnormalities, including additional material of unknown origin at chromosomes, a complex karyotype (near-triploidy), translocations, deletions, monosomies, and trisomies, are highlighted, contributing to a comprehensive understanding of the cytogenetic landscape in hematolymphoid tumors.

Table 4 Recurrent rates of 27 identified cytogenetic abnormality patterns and disease associations among 26 cases of abnormal karyotyping

Recurrent cytogenetic abnormality patterns	Recurrent rate case(s) (%)	Disease-associated
1. Translocation between chromosome 9q34 and 22q11.2 (Philadelphia chromosome)	9 (20.93)	CML
2. Marker chromosome (s)	4 (9.30)	AML, lymphoma
3. Loss Y in male	3 (6.98)	CML, AML
4. Monosomy 21	2 (4.65)	AML
5. Monosomy X in female	2 (4.65)	AML, lymphoma
6. Trisomy 8	2 (4.65)	AML
7. Additional material of unknown origin at chromosome 1p36.1	1 (2.33)	AML
8. Additional material of unknown origin at chromosome 11q25	1 (2.33)	ALL
9. Additional material of unknown origin at chromosome 15q10	1 (2.33)	PV
10. Additional material of unknown origin at chromosome 15q26	1 (2.33)	AML
11. Complex karyotype (Near-triploidy)	1 (2.33)	Lymphoma
12. Deletion of chromosome 1q25	1 (2.33)	Lymphoma
13. Deletion of chromosome 8q22	1 (2.33)	AML
14. Monosomy 6	1 (2.33)	AML
15. Monosomy 8	1 (2.33)	AML
16. Monosomy 10	1 (2.33)	Lymphoma
17. Monosomy 16	1 (2.33)	AML
18. Nullisomy 18	1 (2.33)	Lymphoma
19. Translocation between chromosome 8q22 and 21q22.3 (RUNX1/RUNX1T1 fusion gene)	1 (2.33)	AML
20. Translocation between chromosome 11p15 and 12q15	1 (2.33)	PMF
21. Trisomy 4	1 (2.33)	AML
22. Trisomy 11	1 (2.33)	AML
23. Trisomy 14	1 (2.33)	Lymphoma
24. Trisomy 15	1 (2.33)	CML
25. Trisomy 18	1 (2.33)	Lymphoma
26. Trisomy 21	1 (2.33)	AML
27. Trisomy 22	1 (2.33)	ALL
Total	43 (100%)	

Abbreviations: p, short arm of chromosome; q, long arm of chromosome; CML, chronic myeloid leukemia; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; PV, polycythemia vera; PMF, primary myelofibrosis.

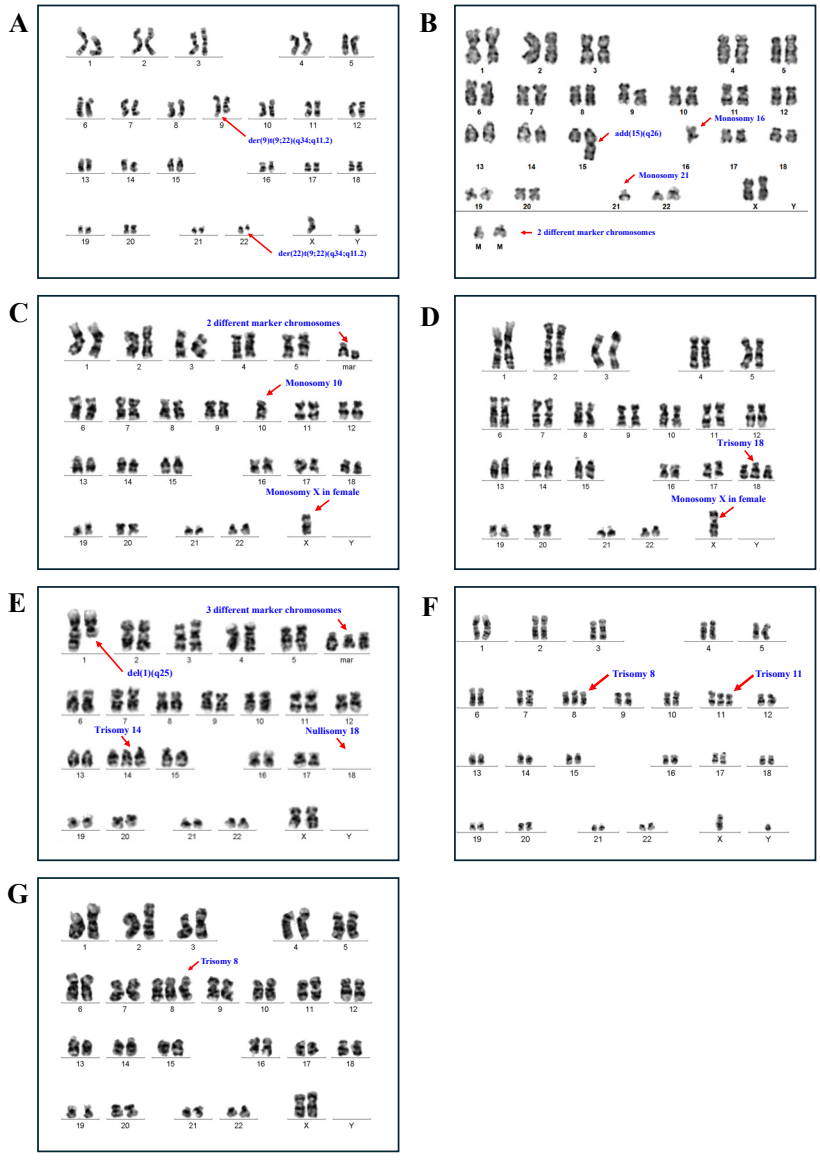

Figure 1 The representative karyotype of cytogenetic abnormalities in hematolymphoid tumors.
(A) The representative of CML reveals a result of translocation of 9q34 and 22q11.2.
(B) The additional chromosome material from an unknown origin was attached to 15q26, monosomy 16, monosomy 21, and 2 different marker chromosomes.
(C) This metaphase cell indicates monosomy 10, monosomy X, and 2 different marker chromosomes.
(D) Monosomy X and trisomy 18 was demonstrated in this metaphase cell.
(E) The deletion of 1q25, trisomy 14, nullisomy 18, and 3 marker chromosomes was represented.
(F) Trisomy 8 and trisomy 11 was showed.
(G) The karyotype revealed a result of trisomy 8.

Note: This karyotype analysis is conducted using GenASIs Bandview software (Figure 1B), and Ikaros Karyotyping software (Figure 1A, 1C-1F).

Abbreviations: add, additional chromosome material from an unknown origin; del, deletion; der, derivative chromosome; mar, marker chromosome; q, long arm of chromosome; t, translocation.

Discussion

The comprehensive investigation of 580 cases in this study, subject to stringent exclusion criteria, has provided valuable insights into the demographics and prevalence of hematolymphoid tumors. The robust dataset of 314 confirmed cases revealed a diverse spectrum of hematologic malignancies, with notable gender distribution and age ranges. Females accounted for 48.70% of the cases, from 0 to 81 years, while males constituted 51.30%, with ages ranging from 2 to 89 years. This indicates that hematolymphoid tumors exhibit heterogeneity in type and age, affecting both males and females globally[19].

Identifying the five most prevalent hematolymphoid tumors sheds light on the epidemiology of these diseases within the studied population. CML emerged as the most common, followed by AML, ALL, MDS, and lymphoma at 2.87%. The difference in incidence between our study and the previous one was observed. Our study found that the most common is CML, followed by AML, ALL, MDS, lymphoma, and other hematolymphoid tumors. On the contrary the survey from Zhang et al. found that the most common hematologic malignancies in 2019 were non-Hodgkin lymphoma, followed by other leukemia, PCM, ALL, AML, CML, and Hodgkin lymphoma. This may be based on the laboratory investigation decision made by the internist in our patients, which did not represent real disease incidents[19]. In addition, the number of newly diagnosed leukemia cases from 1990 to 2017 increased, especially AML and CLL, suggesting that it might become a major global public health in the future[20]. Similar to this study (Table 3), another study observed a higher incidence of CML in males compared to females. In terms of gender, the incidence and death of hematologic malignancies are generally higher in males than in females globally. This may be caused by hormonal, genetic, and environmental factors and requires further study[19].

Importantly, our study focused solely on karyotyping using bone marrow specimens, which may not precisely represent the incidence of hematolymphoid tumors. The karyotype analysis further enriched the understanding of hematolymphoid tumors by categorizing abnormal karyotypes into specific diagnostic groups. Among the 26 cases displaying cytogenetic abnormalities, CML and AML were predominant, reinforcing the association of these chromosomal aberrations with specific hematologic malignancies[8,21]. These findings underscore the heterogeneity of genetic alterations and their potential implications for disease classification and targeted therapeutic strategies in this patient population.

We differentiated the cases into diagnostic groups, leading to each treatment line according to the 27 recurrent cytogenetic abnormality profiles demonstrated among the 26 cases with cytogenetic abnormalities in this study. In our research, CML also became one of the hematolymphoid tumors with abnormal cytogenetic evaluation. The abnormally small chromosome, der(22)t(9;22)(q34;q11.2), also known as the Philadelphia chromosome, was the first recurrent cytogenetic abnormality found in CML as well as hematolymphoid tumors[12, 22]. Imatinib, a tyrosine kinase inhibitor (TKI), is the first targeted drug therapy for CML patients[11]. Some research presents an adverse prognostic outcome in TKI in specific adverse cytogenetic profiles, described as additional cytogenetic aberrations (ACA). The high-risk ACA group seems to suffer from worse prognostic outcomes compared to the low-risk group[21,23]. According to the management guidelines of CML patients in our practice, the clinical adaptation of the cytogenetic profile was still questionable for treatment choice and risk stratification. The cytogenetic profile could classify the risk group in the AML group to guide post-remission period treatment strategies. Our study demonstrated the cytogenetic abnormality indicating the intermediate and high-risk groups (complex karyotype and monosomal karyotype),

which decided the treatment line of allogeneic hematopoietic stem cell transplantation[24,25]. Complex karyotype in the context of AML is classified as ≥ 3 unrelated chromosome abnormalities in the absence of other class-defining recurring genetic abnormalities, excluding hyperdiploid karyotypes with three or more trisomies (or polysomies) without structural abnormalities[8]. A monosomal karyotype is classified as the presence of two or more distinct monosomies (excluding loss of X or Y) or one single autosomal monosomy in combination with at least one structural chromosome abnormality (excluding core-binding factor AML)[8]. Recurrent cytogenetic abnormalities were also identified in MPN, including PV and PMF. In our country's management guidelines, the cytogenetic feature was not directly involved in the management decision. However, the abnormality contributed to factors of risk stratification in some studies, including the "Three-tiered model Mutation-enhanced International Prognostic Scoring System for PV (MIPSS-PV)"[26]. In one case, our study also detected complex cytogenetic abnormalities in diffuse large B-cell lymphoma. None showed a loss of 17p, indicating a poor patient outcome[27]. A complex karyotype was observed, and the one in the bone marrow specimen strongly affected the prognostic outcome compared to those with a normal karyotype or single karyotypic abnormality[28]. Our study provides information on recurrent cytogenetic abnormality in the northeast Thai population, contributing valuable insights for ongoing and future research endeavors.

Additionally, our study's observation of cases with no metaphase cells and a majority with a normal karyotype highlights the variability in cytogenetic patterns within the studied population. As found in a previous study, the absence of metaphase cells for analysis can occur in hematologic malignancies with high counts[29]. These findings underscore the complexity of hematolymphoid malignancies and emphasize the need for further investigation.

Conclusion

This study thoroughly examined the details of hematolymphoid tumors in the northeast Thai population, covering their demographics, incidence rates, and genetic characteristics. The findings contribute significantly to our knowledge about these cancers, laying the groundwork for improved diagnostic methods and treatment approaches and guiding future research.

Take home messages

In this study of northeast Thailand, chronic myeloid leukemia (CML) emerged as the most prevalent hematolymphoid tumor. Conventional karyotype analysis revealed an 8.28% incidence of chromosome abnormalities, notably the Philadelphia chromosome translocation (9q34 and 22q11.2). These findings inform Thailand's health management strategies for hematolymphoid tumors.

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary

The standard protocol of karyotype in this study will be used as follows.

The process of cell culture

1. Grow cells with 5 mL of RPMI1640 (4:1 ratio of RPMI1640: fetal bovine serum), and incubation at 37 °C in a 5% CO_2 incubator for 24 hours.
2. Add 100 µL of methotrexate (MTX) and incubation at 37 °C in a 5% CO_2 incubator for 17 hours.
3. Centrifuge at 3,000 rpm for 5 min and drain supernatant; after that, add RPMI 1640 6 mL (Repeat steps 3, 2 times).
4. Add 0.01 M thymidine 100 µL and drain supernatant after that incubation for 5-6 hours at 37 °C in a 5% CO_2 incubator.

Harvest cell protocol

1. Add 1 mL of colcemid and incubate at 37 °C in a 5% CO_2 incubator for 15 min.
2. Centrifuge at 3000 rpm for 5 min and drain supernatant.
3. Add 10 mL of 0.075 M KCl and incubate in a water bath at 37 °C for 15 min.
4. Centrifuge at 3000 rpm for 5 min and remove supernatant.
5. Carefully add 6 mL of fresh fixative (3:1 ratio of methanol: acetic acid) to the cells while vertexing.
6. Centrifuge at 3000 rpm for 5 min and remove supernatant.
7. Add 6 mL of fresh fixative to the cells while vertexing (Repeat the step 5 & 6 for two times).
8. The cells are stored at 4 °C for repeating.

The protocol of spread slide

1. Take the cleaned slides and soak them in distilled water at room temperature.
2. Chromosome-harvested culture cells are gently resuspended with pasture pipettes, and a small number of cells are aspirated.
3. Apply 3-4 drops of samples to the slide at the end of the pasture pipette about 1 foot from the slide
4. Wipe the back of the slide dry and place the slide on a heating platform set to 60 °C.
5. Completely write the name and lab number on the slide head.
6. Leave the slides warm for 1 day, and then the slides are dyed.

Staining steps with Trypsin and Giemsa

Trypsin, a proteolytic enzyme, denatures euchromatic histones in DNA regions. Therefore, the regions will appear as bright bands when stained with Giemsa, whereas highly condensed chromatin with a large proportion of histones (protected from trypsin) will be dark bands.

The protocol is the following solutions to 4 Coplin jars.

1. The slides will be treated with trypsin for 6-7 seconds and dipped in the first Coplin jar, composted with 50 mL of phosphate buffer, for 5 seconds. Then, quickly rinse in the jar.
2. Then, leave each slide in the second jar for 8 minutes; this jar contains 50 mL of Giemsa dye (mixing 45 ml of phosphate buffer with Giemsa stock).

3. The third and fourth jars contain 50 mL of dilute water. The slides will be dipped in each jar for at least 30 seconds, and a quick rinse will allow them to dry.
4. Analyze cells with a light microscope under 10X and 100X magnification.

Interpretation

Two independent cytogeneticists collect the data on metaphase cell numbers to evaluate the quantity of chromosome analysis. Metaphase cell numbers are determined at 100X magnification and analyzed using Ikaros Karyotyping software (MetaSystems, Altlussheim, Germany) and GenASIs Bandview software (Applied Spectral Imaging, California, USA). Moreover, the result and interpretation of chromosomes are performed using the International System for Human Cytogenomic Nomenclature System (ISCN). The chromosome abnormality, either numerical or structural, is counted as abnormal.

ORIGINAL ARTICLE

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Possibility of the Modified Wall-stretching Exercises to Improve Shoulder Flexibility

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KEYWORDS
Shoulder limitation; Stretching exercise; Self-management; Rehabilitation; Physical therapy.

ABSTRACT
The shoulder joint frequently faces the risk of joint injury affecting its flexibility due to a large range of motion. Current managements usually require several poses and expert guidance that could affect their clinical application. The present study developed a practical strategy, as the so-called modified wall-stretching exercise, and preliminary investigated its possibility to improve shoulder flexibility in order for the findings to estimate the suitable number of sample size required to confirm the effectiveness of this technique. This quasi-experimental design was conducted in 10 pilot participants (5 females, and 5 males) who had limited flexibility in both shoulders as determined using the back scratch test. Participants performed a modified wall-stretching exercise by placing both hands on the wall and bending their upper trunks downward. They executed this exercise in three hand positions, including turning both hands upward, sideward, and downward with holding for 15 seconds in each hand position. The outcomes of the back scratch test were measured immediately before and after the exercise. Participants demonstrated significant reduction in the distance between both hands of the back scratch test immediately after completing a modified wall-stretching exercise (p-value < 0.01). The preliminary findings suggested the possibility of using a modified wall-stretching exercise as an alternative practical strategy to improve shoulder flexibility. In addition, the finding further suggested that a future study required at least 31 participants to confirm the effectiveness of this stretching protocol.

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Introduction

The shoulder joint has a large range of motion (ROM), enabling the ability to execute daily activities, such as overhead reaching, combing hair, and placing a hand behind the back[1,2]. However, the large ROM also increases the risk of injury affecting joint flexibility, a crucial factor in reducing joint mobility through an unrestricted and pain-free range[3,4]. However, many factors can decrease joint flexibility (e.g., improper joint use, a sedentary lifestyle leading to muscle weakness, poor posture, and age-related systemic decline) that could affect the ability to conduct daily activities[5-7]. Therefore, exercise protocols play crucial roles to maintain and optimize shoulder flexibility.

Currently, many exercise protocols, either active or passive exercises, have been prescribed to maintain and enhance shoulder flexibility[8-11]. However, many of these protocols are challenging for their clinical applications due to the requirement of expert guidance and numerous specific poses that are difficult to remember and time-consuming for their effectiveness[12,13]. With the emphasis on self-care management nowadays, further exploration for a practical and self-administrating strategy may offer an alternative protocol to promote shoulder flexibility.

From the literature review, the researchers found a method of wall stretching exercise that requires an individual to stand facing the corner or the doorway, place both hands on the wall, and lean the trunk forward. However, this exercise protocol can stretch mainly for pectoralis muscles[2,14,15]. When considering particular characteristics of the shoulder, the surrounding soft tissues travel, covering the joint with the attachment between the upper trunk and upper arms. The researchers hypothesized that developing an exercise protocol—as the so-called modified wall-stretching exercise—with fixing the arms on the wall would help fix the distal end (insertion) of the soft tissues. Then, turning the hand positions by leaning the upper trunk downward would alter the soft tissues surrounding the joint to be exposed to the stretching forces of the body weight. Therefore, this study preliminary assessed the possibility of a modified wall-stretching exercise to improve shoulder flexibility. Furthermore, the findings of the present study would be used to estimate the suitable number of sample size required to further confirm the effectiveness of this exercise protocol.

Material and methods

Study design and participants

This quasi-experimental study was conducted with 10 pilot participants, both males and females. All of them had limited shoulder flexibility on both sides, as determined using the back scratch test—both hands could not touch each other—a score less than zero[16,17]. In addition, the eligible participants did not have significant painful shoulder(s) (visual analog scale < 5 out of 10)[18], general joint laxity, or hypermobility, determined through a hyperabduction test[19]or any musculoskeletal or neuromusculoskeletal abnormalities, such as arthritis, cervical radiculopathy, impingement, fractures, frozen shoulder, joint dislocation, or subluxation. This study is part of a research project aiming to preliminary explore an effective and practical strategy to improve shoulder flexibility for caregivers working with individuals with movement impairments (HE662206). Eligible participants signed a written informed consent that had been approved by the Khon Kaen University Ethics Committee for Human Research before participation in the study.

Research protocols

The eligible participants were interviewed and assessed for their demographics, including age, body weight, height, history of shoulder injury, and dominant and non-dominant arms, using the Edinburgh Handedness Inventory[19]. Subsequently, participants were assessed for their shoulder flexibility using the back scratch test for both arms[16,17]. After sufficient rest, participants

performed a modified wall-stretching exercise, with the assessment of the back scratch test thereafter. Details of stretching exercises and outcome measures are as follows.

Modified wall-stretching exercise

In general, a stretching protocol can be done by fixing one end and moving the other end of the soft tissue[20]. Therefore, in this study, participants stood facing the wall at a distance of their arm length. Then, they were instructed to place both hands on the wall firmly at their shoulder level with the elbow extension and the distance between the hands slightly wider than their shoulder width (Figure 1A). The stretching protocol was executed while participants placed their hands in three directions, including upward, sideward, and downward. In each hand position, they were instructed to lean the upper trunk downward as far as they could (Figure 1), but without any discomfort, and hold for 15 seconds[15,21]. Then, they repeated the exercise protocol with the remaining hand positions (sideward and downward, Figure 1C and 1D), and thus, the total stretching duration took approximately 45 seconds.

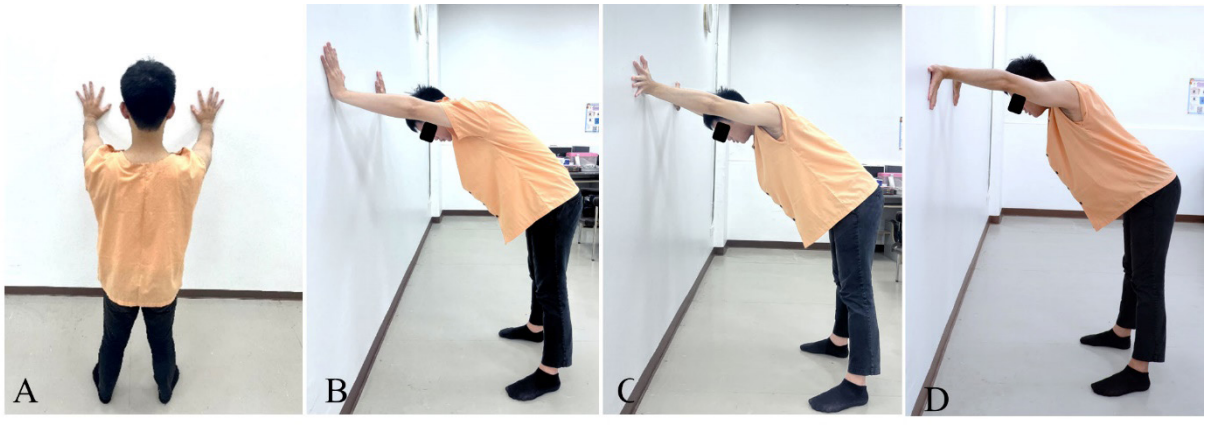

Figure 1 A modified wall-stretching protocol.
(A) Starting position.
(B) Stretching while hands in upward position.
(C) Stretching while hands in sideward position.
(D) Stretching while hands in downward position.

Back scratch test

The back scratch test is practical with excellent reliability measure (ICC: 0.98, 95% CI: 0.97 to 0.98) for assessing the upper limb and upper trunk flexibility[16,17]. In a standing position, participants attempted to touch both hands behind their backs, with one hand reaching downward from over the shoulder and the other hand reaching upward from behind their back. The distance between the fingertips was then measured. Scores (in cm) were assigned for the top arm as zero if the fingertips of both hands could contact each other, a negative value if they were unable, and positive if they overlapped each other. The average result from two attempts was recorded for each hand[16,17]. A previous study reported a minimal detectable change of this test at 1.41 cm[16].

Statistical analysis

The Shapiro-Wilk test was used to assess the normality of the data distribution. Descriptive statistics were employed to explain the demographics

and findings of the study. The paired samples t-tests were utilized to analyze the changes before and after the stretching exercise. The level of significant difference was set at *p*-value < 0.05.

Results

Ten participants (five females and five males), with an average age of 36.20±8.27 years and a body mass index of 28.48±5.74 kg/m^2, completed this study. All of them did not have any previous shoulder injury and did not report any noticeable shoulder pain during exercises.

All participants had limited flexibility as measured using the back scratch test, especially in the non-dominant arm, approximately 15 cm (Figure 2). After exercise, they showed significant improvement for both arms, about 3 cm (*p*-value < 0.01, Figure 2). Then, the researchers took these data to estimate the appropriate number of sample size to further confirm the exercise's effectiveness, with the test's power at 0.9. The data indicated that a future study would require at least 31 participants.

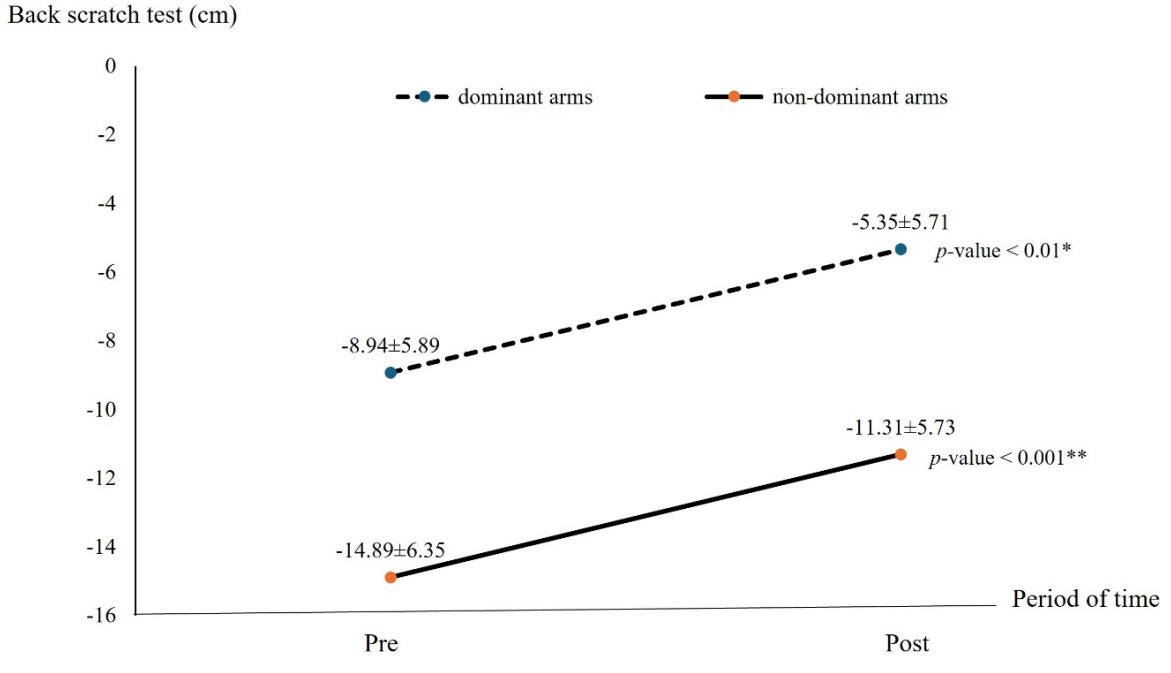

Figure 2 The change of back scratch test of the dominant and non-dominant arms before and after the modified wall-stretching exercise.

Note: The data are presented using the mean±standard deviation with the *p*-value from the paired t-test.

Discussion

This study developed a self-administering stretching exercise protocol, as the so-called modified wall-stretching exercise, and investigated its possibility to promote shoulder flexibility in 10 pilot participants. The findings would also be used to estimate the suitable number of sample size required to further confirm the exercise effectiveness. The results revealed a significant improvement in the back scratch test, approximately 3 cm for both arms, immediately after the modified wall-stretching exercise (Figure 2). This improvement was greater than the level of minimal detectable change of the test (1.41 cm)[16].

The improvement found in the study may preliminary reflect the clinical benefit of the modified wall-stretching exercise. By fixing their hands against the wall and leaning the upper trunk downward (Figure 1), the protocol enabled effective and self-controlled stretch of both ends of the soft tissues in the arms and upper trunk, utilizing the body weight. Then, turning the hands in three directions (upward, sideward, and downward, as depicted in Figure 1) altered the soft tissues surrounding the shoulders, arms, and upper trunk to the stretching force from the body weight. This explanation may be the possible reason for the significant improvement immediately after the exercises (p-value < 0.01, Figure 2).

The back scratch test measures combined shoulder movements, including internal rotation, extension and adduction of the lower arm, external rotation, flexion, and upper arm abduction. Outcomes of the test, therefore, can be used as a quick screening measure for shoulder limitations[22]. In addition, the back scratch test can be used to reflect the flexibility of the anterior compartment of the trunk due to its involvement as the origin of shoulder muscles[23]. Therefore, the improvement in the outcomes of this test preliminary suggested the benefit of a modified wall stretching exercise for the upper limb, particularly the shoulder and upper trunk flexibility.

Previously, some studies have reported the benefit of exercise protocols on the outcomes using the back scratch test. Stanziano et al[24] reported the improvement of the back scratch test for 0.83-1.76 cm after an 8-week static stretching exercise using rope loops. Another study also found significant improvement in the back scratch test after a 12-week Nordic Walking training program in postmenopausal female office workers[25]. A recent study additionally found a significant improvement in the back scratch test after 12-week resistance exercises with low-intensity elastic bands and full body stretching exercise program[26]. With its practicability and self-administration protocol, the present findings suggest the possibility of using a modified wall stretching exercise to promote upper limb and upper trunk flexibility.

Limitations

This single-group pre-and post-test design involved 10 pilot participants who did not have any other problems accompanying joint limitation, such as pain, history of joint injury, or inflammation, with the outcome assessment only using the back scratch test. Thus, the findings may not clearly confirm the clinical utility of this technique for the general population. Therefore, a future study of at least 31 participants who have other problems relating to joint limitation using a randomized controlled trial covering other variables relating to joint functions would clearly confirm the effectiveness of this practical stretching strategy, namely a modified wall-stretching exercise.

Conclusion

This study preliminary supported the effectiveness of a modified wall-stretching exercise for 45 seconds on shoulder flexibility (p-value < 0.001). However, a further randomized controlled trial with at least 31 participants is needed to clearly confirm the effectiveness of this practical and self-administrating exercise protocol.

Take home messages

A modified wall-stretching exercise may be applied as an alternative practical strategy to promote upper limb and upper trunk flexibility.

Conflict of interest

The authors declare no conflict of interest.

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