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H.M. Queen Sirikit Building, Faculty of Medicine, Chulalongkorn University and the Cardiac Center

H.M. Queen Sirikit Building serves mainly the Department of Pediatrics, Faculty of Medicine, Chulalongkorn University and the Cardiac Center. To commemorate the auspicious occasion of H.M. Queen Sirikit's sixtieth birthday on August 12, 1992, the Thai Red Cross Society and Thai people made a concerted effort with boundless rejoicing to contribute to the Queen Sirikit building's construction for Chulalongkorn Hospital under the royal patronage. H.M.Queen Sirikit graciously inaugurated the building on August 26, 1997.

(*Photo by Professor Emeritus Chule Thisyakorn, M.D.*)

Original Article

Mothering and acculturation: Experiences during pregnancy and childrearing of Filipina mothers married to Japanese

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Summary **This study aims to describe the lived experiences of Filipina mothers married to Japanese during pregnancy and childrearing. Eight focus group interviews (FGI) were conducted among 39 Filipino mothers who are currently residing in Japan to obtain significant information with regard to their pregnancy and childrearing experiences. Content analysis was used to extract relevant themes that will describe the experiences of this group of migrant mothers. The findings revealed three major themes: 1) cultural barriers during pregnancy and childrearing; 2) mothering at the different stages of childrearing; and 3) positive adjustments to a new role in a new environment. The establishment of means of communication with migrant mothers effectively enabling them to understand important information for promoting healthy pregnancy and childrearing is strongly recommended. Provision of school information in the English language and enhancing the resilient character of the Filipina are important strategies in promoting positive pregnancy and childrearing experiences among Filipina migrant mothers.**

Keywords: Childrearing, Filipina mothers, pregnancy, lived experiences, intercultural marriages

1. Introduction

In 2006, the Commission on Filipinos Overseas (CFO), an agency of the Philippine Government that upholds the interests and well-being of migrant Filipinos, reported an increase of intermarriages between Filipinas and foreign men. Approximately 25,000 intermarriages were reported that year, thirty-one percent of which were marital unions with Japanese husbands (*1*). Also in 2006, the Ministry of Health, Labor and Welfare of the Japanese government reported a dramatic increase in the number of children of mothers with Philippine nationality. Data further reveal that marriages among Japanese husbands with a Filipina wife reached 12,150, the highest among the recorded international marriages in Japan for that year (*2*). In 2007, there were recorded 1,417 live births of offspring of Japanese-Filipina couples (*3*).

Acculturation and mothering are significant life events for Filipina mothers who are married to Japanese. Filipina mothers of Japanese children are confronted with several issues related to cultural differences and childrearing. The adjustments related to cultural patterns and behaviors are part of the acculturation process that migrants experience when

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they move from country to country. Berry defines acculturation as a process of adjusting to a dominant culture resulting in a modification of one's individual behaviors and becoming embued with the cultural behaviors of the new group or society (*4*). During this process, the migrant mother is plunged into a new culture and undergoes a complex process of assimilation, integration or marginalization. Hoang *et al*. mentioned that culture is strongly associated with a woman's reproductive health beliefs. How care is provided to the unborn and the expectant mother, methods of giving birth, and practices during the pre- and postnatal periods vary considerably with the cultural beliefs and practices of the woman (*5*). The mothering role assumed by a woman upon knowledge of her pregnancy is composed of a mixture of attitudes which she develops through interactions among biological and environmental variables throughout her lifetime. Mothering basically refers to giving birth or caring for someone like a mother, and although most women find this a fulfilling role, the impact of immigration and acculturation still tends to cause extraordinary problems. Studies reveal that a mother's orientation to motherhood changes with acculturation. The demands of transmitting traditional culture and integrating with the culture of the host country during the childrearing process results in unexpected cultural conflict and aggravates the psychological concerns of a migrant mother (*6*).

Several studies reveal that Japanese mothers themselves experience high levels of maternal parenting stress, which greatly influence their child's level of psychological functioning (*7*). A steady rise in the intensity of maternal anxiety about childrearing was reported in 1999. The normal mental and psychological development of a child is thought to be at great risk if there are continuous perturbing experiences during the childrearing stages of mothering. Akazawa stressed the importance of improving the childrearing environment of the Japanese mother to reduce her childrearing anxieties and facilitate nurturing of healthy children (*8*). In a study reporting the confidence of Asian mothers in childrearing, 48% of Japanese women included in the study reported that they were not confident in their ability to rear their child: they also reported degrees of unhappiness and inability to relax while interacting with their child (*9*). A lack of confidence in childrearing has a positive effect on the inability of the mother to adequately care for her child, which is closely related to self-efficacy. On the other hand, Japanese mothers who have more acceptable emotional interactions at home are viewed as having more secure pre-schoolers. Improving the childrearing environment, providing more information and guidance, and establishing support networks were some of the researchers' recommendations.

In February 2002, the Ministry of Health, Labor, and Welfare introduced the Sukoyaka Family 21 Agenda. (The term "*sukoyaka*" means "healthy and happy" in Japanese.) This program was designed to promote a national campaign that would create an environment conducive to healthy birthing experiences and anxiety-free parenting, resulting in good and healthy children. Part 2 of the agenda states that it will assure safety and comfortable pregnancy and childbirth and support for infertility; Part 4 stipulates promotion of children's trouble-free mental development and alleviation of parents' anxiety about childrearing (*10*). Though these relevant programs exist to meet the need for healthy maternity and childrearing in Japan, they do not clearly specify whether or how the same agenda will be provided for pregnant migrant women who have their own special needs and at the same time seek and require positive pregnancy and childrearing experiences. It is the aim of this study to describe the lived experiences of Filipina migrant mothers during pregnancy and childrearing in Japan. The study underscores the roles, difficulties, and coping strategies they utilized to manage their mothering situation. Specifically, it explores the needs identified by these migrant women during pregnancy and childrearing. Furthermore it discusses how Filipina women evaluate their experiences as migrant mothers and provides valuable recommendations to alleviate the problems of migrant women during pregnancy and childrearing. These suggestions are necessary in order to contribute to healthy and happy Japanese families, the goal of the Sukoyaka Family 21 Agenda.

2. Methods

This is a qualitative descriptive study which utilizes content analysis to explore the experiences during pregnancy and childrearing of Filipina mothers married to Japanese. Filipina mothers were asked to participate in scheduled focus group interviews (FGI) using purposive sampling. Krueger defined a focus group interview as a carefully planned discussion designed to obtain perceptions in a defined area of interest in a permissive, non-threatening environment. FGIs are commonly used to obtain general background information about a topic of interest or to generate research hypotheses that can be submitted for further research and testing; they can also serve as new sources of stimulating ideas and creative concepts. In this study, FGIs generated real-life impressions of the participants' pregnancy and childrearing experiences (*11*).

There are also six essential questions that must be addressed when conducting the content analysis process. There questions include: which data are analyzed? how are they defined? what is the population from which they are drawn? what is the context relative to which the data are analyzed? what are the boundaries of the analysis? and what is the target of the inferences?

(*12*). Content analysis starts with word and keyword frequencies, but this process of analysis extends beyond word counts: some words must be analyzed in their specific context to be more comprehensible. This method assumes that the words and phrases that are most often mentioned are those that reflect the major important concerns of the participants in an opportune communication (*13*).

Interpretation and editing of the responses during the interviews were utilized to explore the difficulties, coping strategies, and support available to the Filipina mother as well as the roles that she assumed during pregnancy and childrearing periods. Relevant themes and patterns were identified during the process of data analysis.

2.1. *Participants and recruitment process*

The researcher requested the assistance of the Philippine Embassy in Tokyo to look for groups of Filipina migrant women residing in Japan. A list of organizations with their corresponding contact persons was provided by the embassy, and each group was subsequently contacted by the researchers. The participants were recruited with the help of the different group coordinators of Filipina women's organizations in various areas in Metropolitan Tokyo and nearby cities. There were groups that the embassy strongly recommended because of their active participation in varied embassy activities. The availability and willingness of the participant to take part in the focus group interviews was considered, and purposive sampling was utilized to get the needed number of participants for each interview session. The inclusion criteria for the study were: being a Filipina married to a Japanese husband, having a child from the said marriage, having lived in Japan for at least a year, and being willing to share honestly her experiences as a migrant mother. Participants were contacted by telephone or e-mail, after which initial meetings were scheduled. The researcher joined organizational meetings and church activities with the participants in order to establish rapport and get to know each participant prior to the interview. The schedule for each focus group interview was jointly decided by the researcher and participant to assure attendance at the scheduled FGI.

2.2. *Interview and interview procedure*

Participants who came during the scheduled interview were asked to fill out a consent form and information sheet before the interview. The consent form was translated into Filipino and was made available to those participants who had difficulty understanding the English language. The researcher used the information sheet to collect relevant demographic data about the participants. After ensuring that the participants had read, understood, and signed the consent form, the interview begins. Permission to record the entire interview using an audio tape recorder was solicited before the interview. All interviews were recorded and lasted for about 90 to 120 min. They were conducted in Tagalog, the interviewees' mother language in the Philippines, in order to capture the essence of each of the responses, but participants were encouraged to answer in the language in which they were most comfortable in expressing their ideas. Thus we obtained responses in three languages: Tagalog, English, and Japanese. The following questions were asked during each interview:

1) How would you describe your maternal and childrearing experience?
2) What roles did you assume during childrearing?
3) How were you able to cope with childrearing?
4) What kinds of support did you receive during pregnancy and childrearing?
5) How would you evaluate your pregnancy and childrearing experience?

2.3. *Data analysis*

The researcher used content analysis to analyze the solicited information. The audio taped interviews from the 8 focus groups were transcribed verbatim, printed, and analyzed according to the frequency of similar words, phrases, and sentences to identify prevailing

Table 1. Demographic data on Filipina migrant mothers married to Japanese

Age of participants (years)*	
25-29	2 (5)
30-34	5 (13)
35-39	17 (44)
40-44	9 (23)
45-49	6 (15)
Length of stay in Japan (years)*	
1-4	2 (5)
5-9	9 (23)
10-14	10 (26)
15-19	15 (38)
20-more	3 (8)
Japanese proficiency*	
Good	15 (38)
Fair	23 (59)
Poor	1 (3)
Number of children per participant*	
1	19 (49)
2	11 (28)
3	9 (23)
Age of child (years)†	
> 1-3	8 (12)
4-6	8 (12)
7-12	25 (38)
13-17	21 (31)
18-above	5 (7)

* Total number of mothers is 39; † Total number of children is 67; Data are the *n* (%).

Table 2. Location, dates and number of participants per focus group interview (FGI) (*n* = 39)

Location	Date	No. of participants
#1 Ichikawa	6-Jun-05	8
#2 Yokosuka	30-Jul-05	5
#3 Akabane	3-Aug-05	5
#4 Tokyo	6-Aug-05	4
#5 Goi	15-Aug-05	4
#6 Hiratsuka	20-Aug-05	4
#7 Urawa	21-Aug-05	5
#8 Kasukabe	27-Aug-05	4

themes and sub-themes. Concurrent content analysis was continued until data reached the saturation level and the researchers were able to arrive at a meaningful description of the Filipina mothers' experiences during pregnancy and childrearing. Codes from raw data were formulated and were later categorized. Direct quotes of participant responses' were done to give integrity to the identified essences. Initial codings were provided to other members of the research team for validation and evaluation, after which the findings were confirmed to ensure that the researchers concurred with each other on the credibility of the findings.

The study was approved by the Institutional Review Board of The University of Tokyo and written informed consent was obtained from each participant.

3. Results

3.1. *Demographic data*

Table 1 shows the FGI participants' demographic data, and Table 2 shows the location, date, and number of participants for each FGI. A total of 39 Filipina migrants married to Japanese participated in 8 focus group interviews conducted from June to August 2005.

Forty-four percent (44%) of the respondents were 35 to 39 years of age and had stayed in Japan for 15 to 19 years. More than half (59%) of the participants rated themselves "fair" in Japanese language proficiency, and 38% of the Filipina migrants rated themselves "good" in their Japanese language skills, while 3% admitted that they have a "poor" ability to communicate in the Japanese language. The majority of the participants (49%) have 1 child, 28% have 2 children, and the remaining 23% of mothers have 3 children. Thirty-eight percent (38%) of the participants' children are 7 to 12 years of age and 31% are from 13 to 17 years old.

3.2. *Themes and sub-themes*

Table 3 summarizes the lived experiences of the Filipina migrant mothers during pregnancy and childrearing. We identified three main themes and 10 sub-themes: 1) cultural barriers during pregnancy and childrearing (sub-themes: communication problems, discrimination in the new Japanese family, and clash between two cultures); 2) mothering at the different stages of childrearing (sub-themes: learning childrearing skills, managing children's schooling needs, assisting children's character development, and keeping a close and strong family); and 3) adjusting positively to a new role in a new environment (sub-themes: recourse to Filipinas' resilient character traits, securing significant support from family and friends, and enjoying the life of being a migrant wife and mother).

3.2.1. *Cultural barriers during pregnancy and childrearing*

Filipina migrant mothers in the study mentioned similar difficulties regarding adjustments to Japanese language and culture. These difficulties prevent them from experiencing healthy pregnancy and childrearing.

Communication problems: The lack of Japanese language skills on the part of Filipina migrant mothers and the lack of English skills among hospital personnel is the reason why health instructions that are important during pregnancy and childrearing are not well understood or followed by Filipina migrant mothers. Health classes were given only in Japanese without interpreters, which prevents Filipina mothers from participating in this program.

Mothers also experienced being humiliated when they made mistakes while speaking Japanese and felt frustration because they could not express their ideas accurately.

"I can not express my feelings accurately because of my limited Japanese skills and I can not understand the instructions and terminologies used in the hospital… they are different from the Japanese I hear at home" **G#1M4,G#2M3,G#3M5,G#5M3**

"Lots of times I was laughed at because of my wrong Japanese…I would rather ask my husband to talk for me but when he is not around I am so frustrated," **G#1M3,G#3M5**

Discrimination in the new Japanese family: The majority of the mothers who participated in the study reported feeling discriminated against in their new Japanese family. There were incidents where the husband confined her away from her Japanese in-laws because of their imminent disapproval of the marriage. The Filipina wife also recalled times when she was not introduced as the wife or as the mother of their new grandchild. These situations gave her feelings of being unwanted and unloved.

"during the family's New Year's celebration I was told to stay inside the room and told not to mingle with the other guests; I was not introduced to the other family members, only my son was…" **G#4M1**

Clash between two cultures: In the Filipino and the Japanese cultures, the mother is the one in charge of taking care of the child. But the Filipina mother reported that the Japanese husband had a strong

Table 3. Summary of the lived experiences during pregnancy and childrearing of Filipina migrants married to Japanese

Codes	Sub themes	Themes
- Lots of times, I was laughed at because of my wrong Japanese - The Japanese used inside the hospital is different from what we use at home - Cannot understand hospital terminology and doctors' instructions during pre-natal check-up - Cannot express my feelings accurately because of my limited Japanese skills - When my child got sick I could not explain my child's condition to the doctor because my Japanese is very poor - Nobody speaks to me because I cannot speak Japanese - Because I cannot talk in Japanese and read kanji[1)] my child calls me a "not good mama" - My child asks me something in Japanese and I can't explain it to her; I burst into tears - I cannot read and understand school documents of my child because it's all in kanji[1)],	Communication problems	Cultural barriers during pregnancy and childrearing
- My husband hid me from his parents when I arrived in Japan while I was pregnant - I was not introduced as the wife of my husband during family gatherings - During New Year's celebrations I was told to stay inside the room and not mix with the visitors - My in-laws are afraid that the neighbors will know I am a gai-jin[2)] and Filipina - When we presented our child to my in-laws, I was asked to stay inside the car; I was left alone; I was never invited to go inside my in-laws' house	Discrimination in the new Japanese family	
- I am always looking for ways to balance the two cultures - Confused on how to discipline my child: the Philippine way? or the Japanese way? - I had conflict with my husband about how to raise our child, the Filipino way or the Japanese way - Different character of my child because of the many influences in and outside the house - My child always thinks that she is not "half-half" - having two different cultures	Clash between two cultures	
- I don't know about anything in baby care, I was afraid to touch my baby's umbilical cord - Ask help of the visiting nurse - I always make international call to my mother in the Philippines for guidance and advice - I cannot stop my kids when they play I feel tired my children makes the place I clean dirty again they are always playing, - Everything was really difficult in the beginning with my first child but with the second and third child I was able to handle it very well	Learning childrearing skills	Mothering at the different stages of childrearing
- Must attend PTA meeting all in Japanese - Need to prepare so many things for school requirements and daily "obento"[3)] - Find ways to pay the very expensive school fees; it is very expensive to send children to school - Provide our child money, house, security, and companionship while they still need it - Watchful of the different influences around my child - Give guidance one step at a time to guide my child all the way - They rely on you during the early years in school but they gradually change because they are learning new things and start to become independent	Managing child's schooling needs	
- I always give my children loving advice - Keeping communication open so that I will be able to know how my children feel - Discipline my child so that she will not hurt anybody - Teach my children good values like how to respect other people, especially the aged - Become friends with their children so that they will not lie to us - Watching my child patiently and continually	Assisting child's character development	
- Need to use teamwork and work together to solve any family problem - Need to solve problems together as a family: it builds my relationship with my husband - Need to keep our home whole and intact; this is very important	Keeping a close and strong family	
- Spend time sharing our feelings - When we talk with each other, our relationship becomes closer - Give and take is needed to understand the culture of each country - Make the first move in communicating with other people; start little by little - Use negative and difficult experiences to improve myself and my outlook in life becomes broader - It is a continuous process of adjustment: I have to continue adjusting to so many things in rearing my child and in living in Japan - Bringing up children is very hard work, so I keep fighting, telling myself "ganbare" [4)], and I am able to rear them really well, I think - Even if my husband is always at work, I was able to bring up my child well by myself	Using the Filipina's resilient character traits	Adjusting positively to new role in a new environment
- My husband spends time interpreting important terms - My husband pushed me to study Japanese - I get my strength from my husband, my primary source of support - At first my husband accompanied me to the hospital, but later he let me go by myself for my benefit to learn and be independent - My child went to grade school; he helped me with kanji[1)] I cannot understand - My Japanese friends and the parents of my child's classmates help me in understanding school work and activities - My Filipina friends encourage me to join Japanese class at the city office; we learn Japanese together; it is a lot of fun - My Filipina friends took care of me when I was in the hospital during delivery; they washed my clothes - Without my friends I would die: my friends relieve me from my stress - Learning with my friends, through my friends and my experiences with them, I was able to learn so many things - I made lots of friends, especially with the Japanese - I'm working hard to improve my Japanese skills by joining the language school at the City offices - I'm making more friends by joining the PTA, school, neighborhood cleaning meeting, community activities - Attending Catholic church and Philippine embassy social activities	Securing significant support from family and friends	
- For me it is a life of happiness, being comfortable, experiencing closeness and love - I learned to be thankful for being here for my children for what we have now - The good things I taught her in Japan I was able to share with my family in the Philippines - I learned to trust and respect myself because I realized that when I trust and respect myself others start trusting and respecting you also - I was able to feel how it is to be a wife and mother; it is a very wonderful experience	Enjoying life of being a migrant wife and mother	

[1)] A "kanji" is a Chinese character. But in the story of this Filipina mother, it includes both Chinese characters and the Japanese kana phonetic syllabary; [2)] A "gai-jin" is a foreigner in Japan. This word can have a discriminatory connotation; [3)] An "obento" is a Japanese homemade packed lunch or snack prepared for schoolchildren to bring from home to school; literally it means lunchbox; [4)] "ganbare" is a Japanese expression meaning something like "Never give up!"

influence in disciplining their child the Japanese way.
"there were lots of times when I tried to discipline my children and my husband would interrupt me and say to me that we are in Japan and we must follow the Japanese custom… I am confused about how to discipline my child the Japanese or the Filipino way…I am always looking for ways to balance the two cultures but it is difficult." **G#1M2,**

3.2.2. *Mothering at different stages of childrearing*

All of the participants in the study agreed that their primary role is to be a mother to their child and that there are expected changes in this mothering role as they progress through the various stages of childrearing. The ability of the mother to provide care for her child from pregnancy to adolescence is influenced by many factors.

Learning childrearing skills: First-time mothers usually verbalized their lack of knowledge in newborn care. The nurses and midwives in the hospital taught her essential childrearing skills during the new mother's hospitalization. But upon coming home she still feels awkward in performing the newly learned infant care skills. At times, the migrant mother would engage in expensive international calls just to ask her mother in the Philippines for guidance on how to take care of her child:
"this is the first time for me: I don't know how to take care of my baby, especially my child's umbilical cord. The nurse comes to help me clean it." **G#1M3, G#5M3**
"if I don't know what to do with my child, I always make long distance calls to my mother in the Philippines for guidance and advice." **G#1M2**
Those who had toddlers and children of pre-school age spoke of their inability to control the restlessness of their child, which often interrupts her household activities:
"I wanted to finish all the household work but the children keep on making the things I clean dirty again; I don't know how to stop them; I feel so tired." **G#1M5,G#4M3**

Managing the child's schooling needs: The Filipina mother realizes that there are more difficult tasks that she needs to do as her child enters school. All of them mentioned that the compulsory schooling system in Japan is very different from the Philippines because it requires their active participation throughout the school year.

They all said that sending a child to school is very expensive because of the fees and requirements. They particularly mentioned that preparing the daily *"obento"* takes much of their time. An *"obento"* (literally it means lunchbox) is a Japanese homemade packed lunch or snack prepared for schoolchildren to bring from home to school. Mothers usually spend considerable time and energy producing an appealing boxed lunch. They must balance the foods' nutritious content and its aesthetically pleasing arrangements in a partitioned box (*14*).

They feel pressured to assure that school requirements are based on standards and to attend PTA meetings and other school activities and festivals. The Filipina mother feels that her responsibilities change in varied stages of childrearing and that she must be watchful of the different influences surrounding her child; she must provide strong guidance and constant reminders until the time comes that they can manage themselves.
"they rely on you during the early years in school, but they gradually change because they learn new things and meet new friends; then they become independent." **G#1,G#2,G#4,G#5**

Assisting the child's character development: This theme summarizes the responses of the participants when they were asked about their role in childrearing. Most of them mentioned that their role fundamentally revolves around the character building of their child which includes giving necessary loving advice to their child, keeping communication open, disciplining and teaching them the meaning of respect, and providing good companionship to their child are their specific roles during childrearing.
"you are not only a mother: it is important to teach your child good values like respect for other people, especially the aged…" **G#1M4, G#1M2, G#4M2**

Keeping the family close and strong: Though most of the participants find it difficult, the majority of them still believe that they are in charge of keeping their family close and strong. The mother must serve her child and husband with dedication and must facilitate open channels of communication among family members. Teamwork and family consensus must be considered in dealing with domestic concerns.
"it is my role to keep our home whole by serving my child and husband, with dedication; I need to keep my family whole and intact; it is very important: I need to take care of the house." **G#1M3, G#3M5,G#5M3**

3.2.3. *Adjusting positively to new roles in a new environment*

Many participants felt that they were able to adjust to being a Filipina migrant mother in Japan because they had in them their natural Filipino character of being positive and cheerful and because they have a great deal of support coming from their family, husbands, and friends.
"the Filipina, even if she has problems, she's always happy and has a smile in her face: that's why she's able to handle her problems very well. That's the Filipina way." **G#2M4**

Using the Filipina's resilient character traits: It is remarkable how her innate attitude of having a

positive outlook in life and being cheerful helps her in dealing with her difficult migrant situation. She has this strong coping strategy to transform her negative and difficult experiences into opportunities to improve her childrearing skills. Her strong character helps her to succeed in childrearing.

"everything was really difficult in the beginning of childrearing, but I was able to handle everything very well." **G#1M5**

"bringing up my children was very hard, so I keep fighting: "ganbare!" (Japanese for "Never give up!") I am able to rear them really well, I think." **G#1M4, G#6M3**

Securing significant support from family and friends: All of the participants strongly agree that their husband and set of friends provided the needed support in order for them to adjust successfully to their new role and environment. The husband gives them the primary support and encouragement for them to study Japanese.

"first he (my husband) would accompany me to the hospital and other places, but later he pushed me to study Japanese and be independent for my benefit. He was right." **G#1M2, G#3M4**

There are two sets of friends for the Filipina migrant women: the Filipino friends and the Japanese friends. The Filipina friends who themselves have Japanese husbands give her the needed advice and support during pregnancy, delivery, and all throughout the childrearing stages.

"my Filipina friends encouraged me to take the free Japanese classes at the city office; they washed my clothes and stayed with me during my delivery; without my friends, I would die." **G#1M2,G#2M5M2M7,G#3M4,G#4M4**

The Japanese friends are usually parents of her child's classmates, and this helps her to understand school-related activities. They provide a broader network where the Filipina mother can learn and understand meaningfully the Japanese language and culture.

"I make lots of friends, especially with the Japanese: this helps me improve my language skills and helps me understand my child's school activities, such as in the PTA." **G#1M4**

Enjoying the life of being a migrant wife and mother: All of the participants reported that their experience during pregnancy and childrearing in Japan was filled with difficulties and hardships. But when asked how they would evaluate their life now, the majority of them responded that they are happy and satisfied.

"it is a life of happiness, being comfortable and experiencing closeness and love of my family..." **G#1,2,3,5,7,8**

They feel that all the painful sacrifices they had were replaced by the joys and happiness of having a child and family.

"I was able to feel how it was to be a wife and mother: it was very wonderful. I never realized how wonderful this experience was: I am so thankful that I was able to survive the challenges of caring for my child and family." **G#6M4**

4. Discussion

We described the lived experiences of Filipina migrant mothers during pregnancy and childrearing in Japan based on the categories that emerged from the focus group interviews. The study revealed that the Filipina mothers of Japanese children experienced hardships during pregnancy and childrearing that were primarily associated with adjustments to Japanese culture. Their lack of Japanese language skills limits the migrant's capacity to receive the necessary health instructions in this vulnerable situation and to give full explanations of her child's condition during consultation or illness. A study conducted among Japanese mothers residing in the United Kingdom reported the same language problems. In that study, Japanese mothers experienced frustration from language difficulties, which caused stress, leading to a sense of dependence and lowered self-esteem (*15*). Another study showed that foreign-born mothers, especially those who could not speak the local language, had higher levels of depression and anxiety than native-born mothers (*16*). Evidence from several studies has revealed how communication influences the health provider's clinical decision making, and that patient-provider interactions have subsequent effects on health outcomes. In this case the perinatal and childrearing periods put the Filipina migrant mothers at great risk.

The discrimination among Filipina wives is related to the stigma attached to Filipina women's working in entertainment bars and women wanting to marry "rich" Japanese men to escape their poverty in the Philippines (*17*). Also, the "*ie*" system established during the Meiji period influenced this discriminatory practice toward women and foreigners. The Japanese word "*ie*" literally means "household" or "family" and it involves the residence, households, or a group of families according to the Japanese civil code and the family lineage (*18*). The "*ie*" is a patrilineal system where everything is generally decided by the senior male and women are considered inferior. Women are in vulnerable situations because they have to adjust themselves to the customs of their husbands' "*ie*" and work hard to satisfy their husbands' parents. They have to bear children; the inability to fulfill such obligations results in forced divorce or being sent away. More importantly, this system has two important principles: 1) it puts the family's before one's own needs and 2) it makes a distinction between those in and those outside of one's "*ie*" (*19*). The system was formally abolished with the revision of Japanese family law in 1947,

when Japanese society began a transition to a more Americanized nuclear family system. However, due to the Confucian principles underlying the *ie* concept, the practices are still informally followed to some degree by many Japanese people today (*20*). Japanese society is still influenced by the "*ie*" system as individuals and institutions still clearly discriminate against outsiders as well as sojourners (*18*).

Thus marrying someone outside the established social structure is a major threat to the family or society's homogenous and conservative cultural practices.

The confusion between two cultures experienced by the Filipina mother is due to the clash in the beliefs of the minority culture (Filipina) and the dominant culture (Japanese). Mothers are expected to transmit the cultural traditions and language of their country of origin to their children and to assist their child to adjust to expectations of both cultures. Attempts at integrating both cultures can prove problematic for immigrant women, leaving them vulnerable to misunderstandings and discrimination (*6,21*).

This study was able to elaborate two important features in the lived experiences of Filipina migrant mothers during pregnancy and childrearing. These salient aspects are related to mothering and acculturation.

4.1. *Mothering*

Mothering is a lifelong, multifaceted and vital process of nurturing, which primarily focuses on the growth and development of one's child (*22*). Women coming from different racial groups express their cultural values related to mothering through family roles and structures and through customs and beliefs about child protection and rearing (*6*).

In Japan, women were confined inside the home and their main function in marriage was to raise a son and demonstrate excellence in domestic domains as support for her hard-working husband. The mother is responsible for the child's growth and educational achievement (*7*).

In Filipino families, though the husband is evidently the head, the wife still manages the home and finances. It is the mother's responsibility to provide a cooperative, close, and highly personalized environment where children are brought up to be refined, helpful, humble, and religious (*23*).

Child discipline differs between the two cultures in various ways. Filipina mothers in general discipline their children to teach good values, to mold their child's character and personality, to correct misbehaviors according to the standards of society, and to equip their child with a moral sense of right and wrong. Furthermore Filipino mothers believe that discipline is important in order for their child to grow up healthy, strong, intelligent, and respectful. Spanking (corporal punishment) and reducing school allowances are acceptable forms of punishment in order to discipline and correct the Filipino child's wrong behavior (*24*).

In Japanese society, parental discipline is regulated by the goal of the Japanese family value system. When children begin to show assertive behaviors and noncompliant attitudes in everyday life, mothers are advised not to control these behaviors too strongly, because these are expected to be normal. Japanese mothers seldom confront their child's noncompliance but they indirectly urge their child to comply by focusing on the consequences of their child's misdeeds. When children resist the mother's suggestions, the Japanese mother tones down her control attempts and sometimes gives in to their child's desires (*25*).

The focus of discipline among Filipino children is somewhat similar to Americans' disciplinary attitudes, which focus primarily on the "fear of being punished"; while Japanese children are made aware of the impact of their behaviors on others: they are brought up to be more concerned about how the other child would feel (*26*).

The Philippines' long history of Western colonization tends to strongly influence the behaviors of Filipina mothers in childrearing. To date, there are limited studies on child development of Filipino children, but there are studies that reveal Filipina mothers' great concern with values associated with their child's achievements, and they discipline their child strictly in order for them to excel in varied endeavors. They value qualities associated with being talented, smart, hard-working, goal-directed, and responsible. Filipina mothers believe that the achievements of their offspring will bring prestige and economic security to the family. In the case of Japanese, mothers utilize childrearing strategies that are adaptive to their children's signals and that allow children the freedom to learn with minimal interventions. Japanese mothers also encourage emotional autonomy, while Filipina mothers strive for instrumental autonomy (*27*).

Filipina migrant mothers bring with them these internalized roles and perform them as they assume their mothering roles in Japan. The Filipina mother's priority is to manage the home and child's growth and development. The sub-themes under mothering at the different stages of childrearing substantiate the impact of immigration on mothering that has been demonstrated in previous studies.

This study underscores the seven cornerstones involved in developing mothering skills identified by Flaherty (*6,28*). The seven caring functions are managing, caretaking, coaching, assessing, nurturing, assigning, and patrolling. Participants in this study were able to identify functions similar to Flaherty's as they performed childrearing roles. Managing the childrearing needs and keeping the home intact displays the firm commitment of the Filipina mother to the

safety, growth, and development of her child.

Mercer (1995) mentioned that women may struggle with issues related to self-concept/self-esteem, as well as mental and physical health, during these significant stages of childrearing, but most women develop a positive image of themselves over time (*6*). It is during this time that they need to get the necessary confidence and support primarily from the husband, as well as from the visiting nurse, Filipina friends, and even from the family in the Philippines; support from these sources helps her develop the competence to perform her role as a new mother (*29*).

The experience of gratification and joy during pregnancy and childrearing of the Filipina mother is the result of satisfaction, enjoyment, reward, and pleasure that a woman experiences in interactions with her infant and in fulfilling the tasks of the role (*29*). Gratification from mothering is a motivator to persevere in the role.

4.2. *Acculturation*

Filipina migrant mothers undergo a continuous process of adjustment from the moment they set foot in Japan. This demanding process of positively adapting to their new role as mothers in their new home in Japan can be attributed to acculturation: a term that is both a status and a process. Ryder (2000) describes acculturation as the changes in identity, attitudes, values, and behaviors that accompany an individual's movement from their original or "heritage" culture towards a new and different "mainstream" culture (*30*), while another author defined it as a process whereby immigrant groups adopt the cultural practices, traditions, and values of the host country (*31*). For the Filipina migrant, it is both: her successful adaptation in the Japanese culture is a result of a continuous process of adjustment. She believes that she needs to change for her child and family, and this positive attitude originates from the unique Filipino character of resiliency.

The various calamities and revolts that the Filipinos faced as a people were opportunities to assimilate any civilization and thrive in any climate. This allows the Filipino character to be sturdy and resilient. During difficult times, Filipinos demonstrate their ability to bend without breaking similar to a bamboo tree. It is this resilience that enables Filipina migrant mothers to adapt to any difficult situations while overseas and tolerate the loneliness of being far from their families; it is a very evident Filipino coping strategy (*23*,*32*).

Limitations of this study included the size and composition of the participants: the sample was very small compared with the total number of Filipina migrant mothers in Japan, and most surveyed mothers came predominantly from the middle class. This limits the applicability of the results of the study to a bigger population. There is a need to include a bigger number of participants and to use appropriate random sampling to improve the generalizability of the findings. Though we have a considerable span of marital longevity and age range, we failed to focus on the experiences according to the migrant's length of stay in Japan, age upon migration, parity, and life stage, which may have influenced the lived experiences of the participants. Application of varied forms of data collection such as participatory observations might reveal significant findings. In addition, we have only considered healthy pregnancies and childrearing experiences; it is possible that mothers who had a complicated pregnancy and their children might have different experiences from the mothers studies here. Despite these limitations, we find this study helpful in improving health care advice and instructions given to foreign migrant women during pregnancy and childrearing. It also provides a better understanding of the unique coping strategies of Filipina migrant mothers.

5. Conclusions and Recommendations

In conclusion, the lived experience of Filipina migrant mothers during pregnancy and childrearing is a life lived in a balance between mothering and acculturation. Exploring their experience in being new mothers in Japan is vital in order to elucidate and comprehend the difficulties and coping strategies they employ to adapt successfully to their new role in a new environment. The use of a qualitative approach provides participants with an opportunity to express their thoughts and ideas freely without the restrictions of a structured questionnaire. Findings from this study can directly impact health care during pregnancy and childrearing stages and will facilitate reviews of existing health programs to allow more migrant mothers to benefit from them.

This study would like to recommend: 1) having more health/school personnel conversant in English and health/school instruction materials available in a common language; 2) organizing a parent-partner or parent-tandem might be considered helpful in creating support networks for the migrant mothers; 3) and providing a counseling desk at the city office or school for international marriages to help limit confusion among couples in rearing a child having parents coming from two different cultures. Guidance sessions on culture clarification and appreciation can be beneficial to both parents as well as to their children, and it is necessary to enhance the innate cheerful and positive characteristics of Filipina mothers through social activities in hospitals and schools to increase migrant mothers' self-esteem.

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Original Article

Isolation and characterization of anti-T-antigen single chain antibodies from a phage library

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Summary **T-antigen (Galβ1-3GalNAc-Thr/Ser) also known as Thomsen-Friedenreich (TF) antigen is the core 1 structure of *O*-linked mucin type glycans. In normal epithelium, the disaccharide structure is masked by terminal carbohydrate moieties, but is uncovered in most primary and metastatic epithelial malignant tumors. For the purpose of establishing cancer diagnosis and therapeutics, anti-T-antigen antibodies were isolated from a phage library displaying human single chain antibodies. A strategy similar to the previously published method (Sakai *et al.* Biochemistry. 2007; 46:253-262) was used to screen T-antigen specific antibodies, except that a different type of glycolipid was used for panning and screening. Eleven phage clones were isolated and characterized by DNA sequencing and ELISA, which revealed 4 groups of clones with T-antigen binding activity. One single chain antibody (scFv) protein, derived from phage clone 1G11, was expressed in *Escherichia coli* and purified to near homogeneity by two column chromatographies. ELISA and surface plasmon resonance analyses revealed that the purified 1G11 scFv bound to the T-antigen moiety of the neoglycolipid used. This study not only demonstrated the validity of our previously introduced strategy employing the phage display technology in constructing human scFvs against various carbohydrate antigens, but also provided us with various scFv genes that can lead to future development of antibody-based therapeutics.**

Keywords: Human antibodies, Thomsen-Friedenreich (TF) antigen, T-antigen, single chain antibodies, neoglycolipids

1. Introduction

T-antigen (Galβ1-3GalNAc-Thr/Ser) also known as Thomsen-Friedenreich (TF) antigen is the core 1 structure of *O*-linked mucin type glycans. In normal epithelium, the disaccharide structure is masked by addition of carbohydrate chains to form branched and complex *O*-glycans. T-antigen has been identified by immunohistochemical techniques in most adenocarcinomas of the colon, breast, lung, bladder, endometrium, and ovary, as well as during embryogenesis (*1-5*). T-antigen is also known to be associated with cancer invasion (*6,7*). Anti-T-antigen human antibodies would thus provide excellent tools for not only cancer diagnosis but also cancer treatment.

While immunization with carbohydrates often leads to a primary IgM response and no response in some cases because many carbohydrates are self-antigens (*8,9*), phage display technology allows one to generate antibodies against self-antigens. This technology

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has been used mostly to generate antibodies against proteins, whereas its use for carbohydrate antigens has been limited. Previous studies utilized glycoproteins, heteroglycans, and carbohydrate-BSA conjugates as antigens to present carbohydrate-moieties for the production of anti-carbohydrate antibodies by phage display methods (*10-13*). We have adapted this phage-display technology to generate human single chain antibodies (scFvs) using neoglycolipids as antigens, and reported isolation of anti-mannotriose (M3) scFvs from a newly constructed phage library with a large repertoire as a result of CDR shuffling and VL/VH shuffling with the use of unique vector constructs (*14,15*). In the previous study, M3-dipalmitoylphosphatidyl ethanolamine (DPPE) was used as a model antigen neoglycolipid for panning and screening (*14*).

A strategy similar to the previously published method (*14*) was used to obtain T-antigen specific antibodies in this study, except that a different type of glycolipid was used for panning and screening. Galβ1-3GalNAcα-hexaethylene glycol-3,5-bis-8-dodecyloxy benzamide (T-antigen E6-BDB) contained hexaethylene glycol (E6) as a spacer that was expected to reduce non-specific binding of and to increase accessibility to phage antibodies. Its lipid anchor, bis-dodecyloxy benzamide (BDB), was also different from DPPE which was used for the previous studies. From eleven phage clones isolated and characterized, one scFv protein was expressed in *E. coli* and purified to near homogeneity by two column chromatographies. ELISA and surface plasmon resonance analyses revealed that the purified scFv bound to the T-antigen moiety of T-antigen E6-BDB. This study thus demonstrated the validity of our previously introduced strategy employing phage display technology in constructing human scFvs against various carbohydrate antigens, and provided anti-T-antigen scFv genes for future development of cancer therapeutics.

2. Materials and Methods

2.1. *Materials*

Escherichia coli strains used were the suppressor strain TG1 and the non suppressor strain Top10F' form invitrogen (Carlsbad, CA, USA). Helper phages M13KO7 were from GE Healthcare UK Ltd. (Buckinghamshire, UK). Bovine serum albumin (BSA), Human serum albumin (HSA), DPPE, and ABTS/H_2O_2 were from Roche Diagnostics (Mannheim, Germany). Of the neoglycolipids used, both T-antigen and Tn antigen-containing glycolipids (*16*) were synthesized in a laboratory at the Noguchi Institute (detailed procedures will be published elsewhere). Lacto-*N*-tetraose (LNT; Galβ1-3GlcNAcβ1-3Galβ1-4Glc), lacto-*N*-neotetraose (LNnT; Galβ1-4GlcNAcβ1-3Galβ1-4Glc), lacto-*N*-fucopentaose I (LNFPI; (Fucα1-2)Galβ1-3GlcNAcβ1-3Galβ1-4Glc), lacto-*N*-fucopentaose II (LNFPII; Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc), lacto-*N*-fucopentaose III (LNFPIII; Galβ1-4(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc), and lacto-*N*-difucohexaose I (LNDFHI; (Fucα1-2)Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc) were purchased from Dextra Laboratories (Reading, UK). Various oligosaccharides (LNT, LNnT, LNFPI, LNFPII, LNFPIII, LNDFHI) and DPPE were conjugated by reductive amination as described previously (*17,18*). Oxalic acid and other chemicals were from Wako Pure Chemical (Osaka, Japan). Anti-M13 antibodies were purchased from GE Healthcare Bio-Sciences (Piscataway, NJ, USA) and Exalpha Biologicals (Watertown, MA, USA).

2.2. *Panning procedures*

Methods for construction of phage libraries followed by recombination and generation of secondary phage scFv libraries were previous reported (*14*). The library was subjected to four rounds of panning. All rounds of panning were performed with 96-well plates coated with 500 pmol of T-antigen E6-BDB or 500 pmol of E6-BDB/well. After the 1st panning, subtraction panning was performed with 500 pmol E6-BDB/well. Forty-eight, 24, 12, and 6 wells were used for 1st, 2nd, 3rd, and 4th panning, respectively. Phage selection was basically carried out according to previously published procedures (*14*) with some modifications. Briefly, coating of wells with T-antigen was achieved by applying 50 μL of T-antigen E6-BDB (0.02 mM in methanol) premixed with E6-BDB (0.02 mM in methanol), and drying the solvent at 37°C, followed by incubation with 150 μL of 10 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl (TBS) and 3% BSA at 4°C, overnight. Wells were rinsed 3 times with 200 μL TBS. Fifty μL of phage suspension in TBS containing 1.4% BSA were added to wells, which were then incubated for 60 min at 37°C with continuous rotation. After wells were washed 3 times with 200 μL of TBS, bound phages were eluted by addition of 50 μL of 100 mM triethylamine and incubated at 25°C for 10 min and then neutralized by mixing with 100 μL of neutralizing solution (1 M Tris-HCl, pH 7.4, 3% BSA/TBS = 2:1, v/v) in wells, which had been treated with 3% BSA/TBS. Bound phages were further eluted by addition of 50 μL of 100 mM triethylamine and incubated at 25°C for 20 min and were recovered in the neutralization solution as described above. Eluted phages were used to infect 100 μL of *E. coli* TG1 in logarithmic growth at 37°C for 30 min. Infected bacteria, grown on LB agar plate (ø = 10 cm) (Trypton 30 g, yeast extract 20 g, NaCl 5 g, 1 M NaOH 1 mL, 1 M Mops, pH 7.0/1L) containing, 0.1% glucose, and carbenicilline (50 μg/mL) at 25°C for 48 h, were scraped from the plate using a spreader after addition of 2.5 mL of LB-10 mM Tris-HCl, pH 7.5 (SBS) per plate. One mL of this suspension was inoculated into 40 mL SBS containing carbenicilline

(50 μg/mL) and grown with shaking at 37°C for 2 h. Phages were rescued after addition of 40 μL of helper phages containing 3.5×10^9 cfu and incubated at 37°C for 1 h without shaking, followed by addition of kanamycin (25 μg/mL)/chloramphenicol (10 μg/mL) and incubated with rotation at 25°C for 48 h. Phage particles were concentrated using PEG-precipitation, and dissolved in 700 μL of TBS, 700 μL of 3% BSA/TBS, and 1 μL of Benzonase and incubated at 37°C for 1 h. After centrifugation at 18,000 × g for 5 min at 4°C, 1.5 mL of phage suspensions were recovered and used for subtraction panning. For subtraction panning, 50 μL phage suspension/well were applied to E6-BDB-coated wells, and incubated at 37°C for 1 h. Phages not bound to E6-BDB were recovered and used for 2nd, 3rd, and 4th panning which were carried out in a similar fashion to the 1st panning except for washing conditions. Bacteria picked from single colonies after 4 rounds of panning were grown in 200 μL of SBS containing carbenicilline (50 μg/mL), 2% glucose and 6% glycerol in 96 deep-well plates at 37°C for 2 days with rotation. Twenty μL of the bacterial culture were added to 200 μL of SBS containing helper phages and carbenicilline (50 μg/mL), and incubated at 37°C for 2 h without rotation. SBS/kanamycin/chloramphenicol mixture (300 μL/well) was added to the suspension culture, from which phage suspensions were prepared by incubation at 25°C for 32 h with rotation. After centrifugation at 200 × g for 15 min at 4°C, 100 μL of the supernatants were added to wells containing 100 μL of 3% BSA/TBS, incubated at 37°C for 1 h, and kept at 4°C.

2.3. *Screening of phage clones expressing scFvs directed against T-antigen E6-BDB by ELISA*

Binding ability to T-antigen E6-BDB of the bacterial supernatants containing phages was determined by ELISA. Wells of 96-well plates were coated with 500 pmol T-antigen E6-BDB and 500 pmol E6-BDB/well as described above and blocked by incubation with 150 μL of 3% BSA/TBS at 4°C overnight. Control plates were prepared as above without the antigen. Fifty μL of phage suspension were added to the wells and incubated at 37°C for 1 h. The wells were washed 10 times with 200 μL of TBS. Bound phage antibodies were detected by incubation with horseradish peroxidase (HRP)-conjugated anti-M13 antibody at 37°C for 1 h, after which they were washed 5 times with 200 μL of 0.2% Tween 20/TBS and washed 10 times with 200 μL of TBS. Peroxidase activity was detected by reaction with ABTS/H_2O_2 for 30 min and terminated with 1% oxalic acid. Absorbance was measured with a BIO-RAD plate-reader at 415 nm.

2.4. *Colony PCR and determination of DNA sequences*

Colony PCR was carried out according to the previously published procedure (*14*). Briefly, scFv genes were amplified from respective *E. coli* TG1 colonies infected with phages by PCR with a primer set (forward primer Cm-f: 5'-TGTGATGGCTTCCATGTCGGCAGAATGC T-3', reverse primer g3-r: 5'-GCTAAACAACTTTCAA CAGTCTATGCGGCAC-3'). After preheating at 94°C for 2 min, PCR was carried out using 30 cycles under denaturing conditions at 94°C for 20 sec, annealing at 60°C for 20 sec, and extension at 68°C for 1 min. After purification and confirmation in 2% agarose gel electrophoresis, the resulting scFv genes were subjected to DNA sequencing. DNA sequences of scFvs were determined using a 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

2.5. *Determination of carbohydrate-specificity of phage antibodies by ELISA using synthesized neoglycolipids*

Wells of a 96-well plate were coated by addition of 50 μL of LNT-, LNnT-, LNFPI-, LNFPII-, LNFPIII-, and LNDFHI-DPPE, as well as T-antigen E6- and Tn-antigen E6-BDB (500 pmol)/well and drying the solvent at 37°C, followed by incubation with 150 μL of 3% BSA/TBS at 4°C overnight. The rest of the procedures were carried out as described above for screening of phage clones.

2.6. *Expression and preparation of the 1G11 scFv protein*

The 1G11 phage-infected *E.coli* TOP10F' was cultured in 200 mL of SBS containing 0.4% glycerol, carbenicilline (50 μg/mL), spectinomycin (50 μg/mL), and 1 mM isopropyl-thio β-D-galactopyranoside (IPTG) at 25°C, overnight. Cells were collected by centrifugation at 4,000 × g for 30 min, and solubilized with non-ionic detergent Bugbuster (0.2 g whole cells/1 mL) (EMD chemicals, Inc., CA, USA). The extract was diluted 10-fold by the addition of 20 mM Tris-HCl, pH 8.0, and applied to a DEAE-cellulose column (DE52, Whatman, GE Healthcare) (ø1.5 × 28, 50 mL). The DEAE-cellulose column was equilibrated with 20 mM Tris-HCl, pH 8.0, before applying the sample. Bound proteins were eluted using a 0-1 M NaCl gradient. Fractions eluted from the column were analyzed using SDS-PAGE (4-20% polyacrylamide gel, Daiichi Pure Chemicals, Tokyo, Japan) under reducing conditions, followed by staining with Coomassie Brilliant Blue (CBB). The proteins in a duplicate SDS-PAGE gel were transferred to a PVDF membrane. After blocking with 3% BSA/PBS, the 1G11 scFv protein was detected with an HRP-conjugated anti-E-tag antibody followed by fluorescence development using an ECL detection kit (GE Healthcare). The fractions containing immunoreactive bands were analyzed by surface plasmon resonance (SPR) to determine T-antigen binding activity of the eluates.

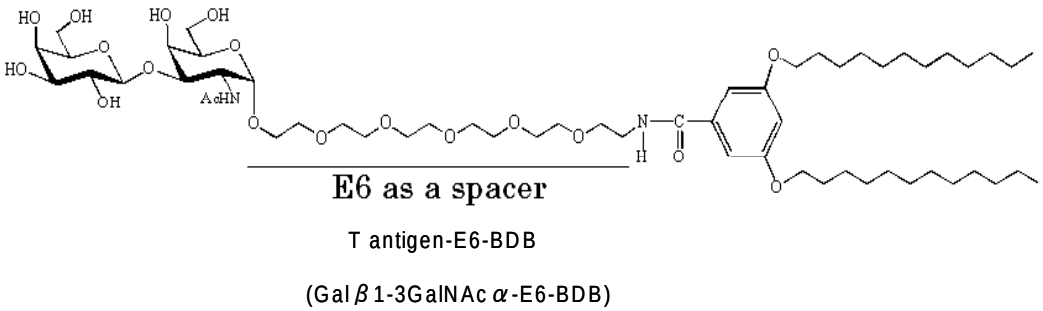

Figure 1. Structure of the T-antigen neoglycolipid used for panning and screening in this study. Galβ1-3GalNAcα-hexaethyleneglycol-3,5-bis-8-dodecyloxy benzamide (T-antigen E6-BDB) is illustrated.

Fractions #21 to #26 which showed immunoreactivity to anti E-tag and binding activity to T-antigen were combined. To this solution, urea and 200 mM imidazole buffer were added to adjust final concentrations to 2 M and 20 mM, respectively. The DEAE-cellulose eluates were then incubated with 2 mL of Ni^{2+}-Sepharose gel (GE healthcare) on a rotator, overnight at 4°C. The gel was packed in a column, from which the passed-through fractions were collected. After the column was washed with 40 mL of PBS containing 40 mM imidazole, the 1G11 scFv protein was eluted with 400 mM imidazole/PBS. Protein concentrations were determined using the BCA protein assay (Thermo Fisher Scientific, Waltham, MA, USA) with BSA used as a standard.

2.7. *T-antigen binding analysis of scFv proteins by surface plasmon resonance*

SPR analyses were performed at 25°C. All solutions were freshly prepared, degassed and filtered through a 0.45 μm pore filter. Binding properities of DEAE-cellulose eluates were determined by SPR using BIAcore 3000 (GE Healthcare). Immobilization on a CM5 sensor chip was carried out using amine coupling at pH 4.5 for 14 min during activation according to the manufacture's instructions. T-antigen-HSA (Dextra Laboratories) and HSA (Sigma) were immobilized at 100 μg/mL onto a positive flow cell and a control flow cell, respectively. The binding analyses were carried out with 10 mM Hepes, pH 7.4, containing 150 mM NaCl and 0.005% Tween 20 at a flow rate of 20 μL/min. The specific binding of the sensorgrams was obtained by subtraction of the control flow channel sensorgram from that of the positive flow cell.

3. Results

3.1. *Screening of scFv-displaying phages against T-antigen E6-BDB*

A phage library displaying human scFvs was subjected to four rounds of panning as previously described using a neoglycolipid (*14*). The structure of the antigen neoglycolipid used in this study for panning and screening is shown in Figure 1. Of 96 clones screened by ELISA using T-antigen E6-BDB as an antigen, there were 24 positive clones with a S/N of > 3 (Table 1). Of those, 11 positive clones were confirmed to encode scFvs by PCR and DNA sequencing. DNA sequencing of scFv sequences of 11 clones revealed deduced amino acid sequences of VH and VL chains as shown in Table 2A and 2B, respectively. Those scFvs were categorized into four groups based on their amino acid sequences (Table 3). Three Group 1 clones, 1E8, 1F6, and 1H7, were found to be identical. Two Group 2 clones, 1E6 and 1F4, shared homology in their heavy chains but differed in their light chain sequences. In contrast, three Group 3 clones, 1E10, 1F1, and 1F9, showed homology only in their light chains. Two identical Group 4 clones, 1G11 and 1H11, were found to be an incomplete scFv without CDRs in their light chains. One additional clone, 1F5, did not show any amino acid sequence homology to those of other clones.

Table 1. Screening of phages displaying scFvs having affinity for T-antigen E6 BDB

	Number of clones
Screened by ELISA	96
ELISA-positive (signal/noise ratio: > 3)	24
DNA sequencing-confirmed scFvs	11

3.2. *Characterization of phages displaying scFvs screened by T-antigen E6-BDB*

Figure 2 shows specificity profiles of phage antibodies, 1E8, 1F6, 1E10, and 1G11, representing Group 1, 2, 3, and 4, respectively, as determined by ELISA using a set of neoglycolipids. A Group 4 clone, 1G11, had the highest specificity and affinity for T-antigen. In contrast, other Group 3 clones showed cross-reactivity to Tn-antigen E6 BDB and LNFPIII-DPPE. Thus, 1G11 was chosen for further characterization.

3.3. *Expression and purification of the 1G11 scFv protein*

E. coli Top10F′ infected with the 1G11 phage was

Table 2A. Deduced amino acid sequence aliment of VH chains of selected 11 clones

Group	clones	FWR1	CDR1	FWR2	CDR2
1	1E8, 1F6, 1H7	QVQLQQSGAEVKKPGASVKVSCKASGYNFA	S-HNMH	WVRQAPGQGLEWMG	IMK--PDNGRSRQTQKLRG
2	1E6	QVQLVESGSELKKPGASVKVSCTASGYTFN	N-YAMN	WVRQAPGQGLEWMG	YIN--TNTGKSTYAQGFTG
	1F4	....QQ.....	.-....	.....	.....
3	1E10	QVQLQQWGAGLLKPSETLSLTCAVSGGSLN	-GYYWN	WIRQPPGKGLEWIG	EIN---HSGSTNYNPSLKS
	1F1	....VES.G.VVQ.GRS.R.S..A..FT..	N-.GMQ	.V..A.....VA	L.S--HAGSDKY.GD.VKG
	1F9	......S..EVKR.GSSVKVS.KA...TFS	ST.AIS	.V..A..Q....M.	G.I--PR..TAKSAQN.QG
4	1G11, 1H11	QVQLQESGPGLVKPSETLSLTCAVSDYSVS	SDYFWG	WIRQSPGRGLEWIG	SIY---HSGSTYYDPSLKS
Other	1F5	QVQLLETGGGLVKPGGSLRLSCAASGFTFI	D-AWLS	WVRQAPGKGLEYIG	RIKKKKDGGTTDYAAPVKG

clones	FWR3	CDR3	FWR4
1E8, 1F6, 1H7	RLTLTRDTSTRTHYMELRNLKSEDTAIYYCAR	DSK----------FFGS--DY	WGQGTLVTVSS-------
1E6	RYVLSLDTSASTAYLQISSLKAEDTAVYYCAR	DSAG--------THRDL--DF	WGQGTLVTVSSGSASAPT
1F4	.....	....---------.....-..	..........-------
1E10	RVTMSVDTSRNQFYLKLDSVTAADTAVYYCAR	---G--------YYKR---DY	WGQGTLVTVSS-------
1F1	.F.I.R.N.KNTV..QMS.LR.D.....	DVVR--------SGDD----L	..........-------
1F9	...ITA.E.TSTV.ME.S.LRSD.....	DPG.TGAVNSYY..YGM--.V	..........-------
1G11, 1H11	RITISMDTSQNQFSLELRSLTAADTAVYFCAR	EKPGWHYYDSGTYYSSGAIDS	WGQGTLVTVSS-------
1F5	RFTISRDDSKSTLYLQMNSLKTEDTALYYCTT	DLLG--------GHDWN--CK	WGQGTLVTVSS-------

Table 2B. Deduced amino acid sequence aliment of VL chains of selected 11 clones

Group	clones	FWR1	CDR1	FWR2	CDR2
1	1E8, 1F6, 1H7	QSVLIQPP-SVSGSPGQSITISC	TGIGSDVWKLNL---VS	WYRQYPGKAPKLLIY	QGRRRPS
2	1E6	DIVLTQTPDSLAVSLGETATINC	KSSQSLLYDSNRRQYLA	WYQQKPGQSPKLLIY	WASTREA
	1F4	QPG.....-.VS.AP.K..S.T.	E--GNNIGSK.----VH	........A.L.V..	YD.DRPS
3	1E10	DIVLTQTPGTLSLSPGERATLSC	RASQSVGN-----TYLA	WYHQKPGQAPRLIIY	GASTRAT
	1F1	E.....S.....	......TG-----N...	..Q.....	...S...
	1F9	E.....S.....	......SS-----R...	..Q.....	...S...
4	1G11, 1H11	SYELMQPP-SLSVSPGQTARI--	----------	----------	-------
Other	1F5	DIQVTQSPSSLSASVGDTVTITC	RASQSVGD------WVA	WYQLKPEGGPKSLVF	ASSAVQN

clones	FWR3	CDR3	FWR4
1E8, 1F6, 1H7	GISDRFSGSKSGNTASLTISGLQAEDEADYYC	SSYRS--SSTWV	FGGGTKLTVL
1E6	GVPDRFSGSVSGTDFTLTINSLQAEDVAVYYC	LQYYR----SLN	FGPGTKVDIK
1F4	...K.....N..NTA....SRVE.G.E.D...	QVWQSNIDHPRV	..G...LTVL
1E10	GIPDRFSGSGSGTDFTLTISRLEPEDFAVYYC	QQYGS---SQYT	FGQGTKVEIK
1F1	.............V.....	.....---.PW.	.....
1F9	.....	....NP--.TW.	.....
1G11, 1H11	----------	----------T	FGQGTRLEIK
1F5	GVPSRFSGRGYGTDFTLTISDLQPEDSATYYC	QQYDD---LPIT	FGQGTRLEIK

Table 3. Eleven clones with affinity for T-antigen are categorized into 4 groups based on their amino acid sequences

Group	Clone names	Primary structure of scFvs (Table 2)
1	1E8, 1F6, 1H7	Three clones are identical.
2	1E6, 1F4	VH domains are nearly identical while VL domains differ.
3	1E10, 1F1, 1F9	VL domains are nearly identical while VH domains differ.
4	1G11, 1H11	Two clones are identical, but lack most of the VL domain.
NA	1F5	This clone is different from others although significant homology to CDRs of other clones is present.

Clones underlined, 1E8, 1E6, 1E10, and 1G11, were used for further studies.

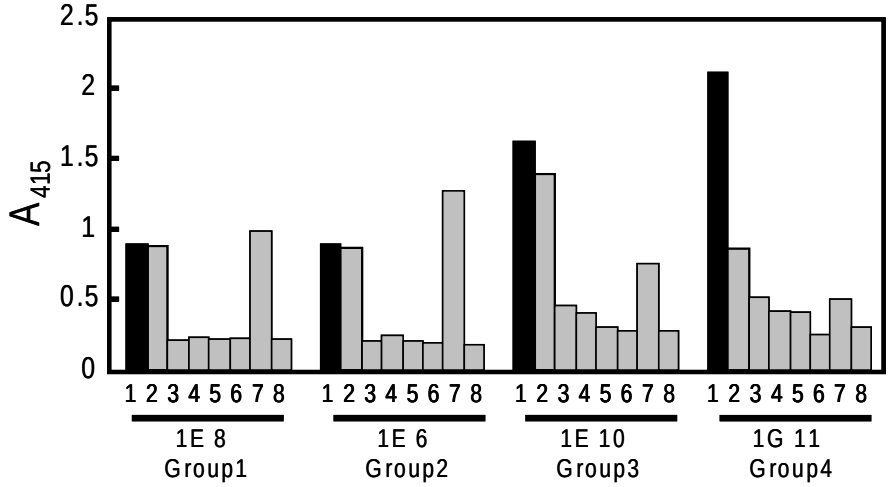

Figure 2. Binding activities of 4 phage antibodies, 1E8, 1E6, 1E10, and 1G11 representing Group 1, 2, 3, and 4 (Table 2), respectively. Various neoglycolipids in addition to T antigen-E6-BDB were immobilized in wells of a 96-well plate. Shown are binding of each phage antibody to T-antigen-E6-BDB (1: ■), Tn-antigen-E6-BDB (2), LnT- (3), LnNT- (4), LNFPI- (5), LNFPII- (6), LNFPIII- (7), and LNDFHI-DPPE (8).

cultured in the presence of IPTG to induce scFv protein expression. Cells collected were solubilized with a non-ionic detergent, from which the1G11 scFv protein was purified by DEAE-cellulose and Ni^{2+}-Sepharose affinity chromatographies. The scFv protein was eluted from a DEAE-cellulose column by a 0-1 M NaCl gradient. Eluted fractions were analyzed by SDS-PAGE followed by CBB staining and Western blotting with anti-E-tag antibody. As shown in Figure 3A and 3B, respectively, the results indicated that fractions 21 to 26 contained the anti-E-tag detectable 1G11-scFv protein. Fractions 21 to 26 were combined and then subjected to SPR analyses using T-antigen conjugated human serum albumin (T-antigen HSA) (Figure 4A). The combined DEAE-cellulose fractions revealed higher binding activity to T-antigen HSA than the crude cell lysates which had been applied to the DEAE-cellulose column (sensorgrams a and b in Figure 4A, respectively). The 1G11 scFv fractions eluted from the DEAE-cellulose column were subsequently purified by Ni^{2+}-Sepharose affinity chromatography. Under non-denaturing conditions, however, 1G11 scFv did not bind to Ni^{2+}-Sepharose gel (data not shown), which suggested that the His-tag located at the C-terminus of 1G11 scFv may not be accessible to Ni^{2+} due to possible steric hindrance. Alternatively, 1G11 scFv was purified by Ni^{2+}-Sepharose chromatography in the presence of 2 M urea. The 1G11 scFv protein was purified to near homogeneity as revealed by SDS-PAGE/CBB staining (Figure 3C) and Western blotting with anti-E-tag antibody (Figure 3D). The yield of the purified 1G11 protein was 85 μg from 3.56 mg (wet weight) of *E. coli* or 312 mg protein of the solubilized fractions.

3.4. *Binding activities of the purified 1G11 scFv protein*

The binding activity of the purified 1G11 scFv protein was determined by ELISA. As shown in Figure 4B, ELISA was carried out with serial dilutions of the purified 1G11-scFv using T-antigen E6-BDB, Tn-antigen E6-BDB, E6-BDB, and BSA. The results indicated that the purified 1G11 scFv has higher binding activity for T-antigen than Tn-antigen.

4. Discussion

In this manuscript, isolation and characterization of anti-T-antigen scFvs were described. Of 96 phage clones screened from the phage library, 11 clones were shown to carry scFv sequences and had affinity for T-antigen E6-BDB, a carbohydrate probe presenting T-antigen. Of note is that a significantly high ratio of positive clones, 11.5% of clones screened, was obtained. When anti-M3 scFvs were previously isolated using M3-

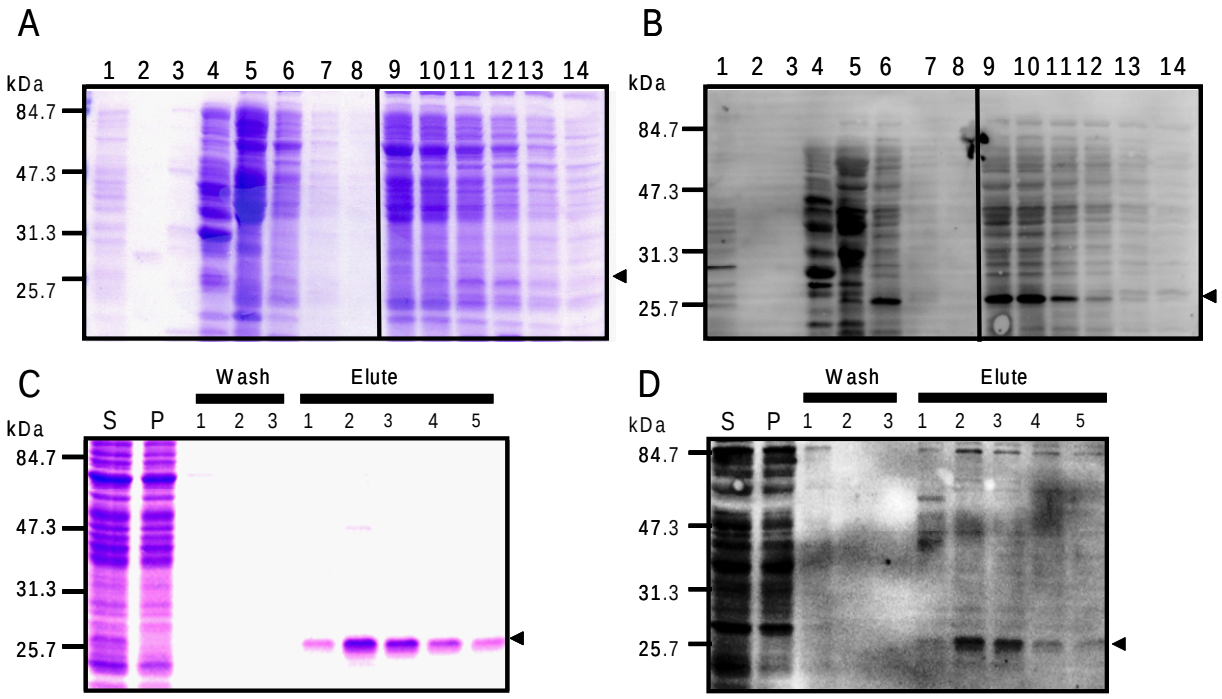

Figure 3. Analyses of the 1G11 scFv protein fractions eluted from DEAE-cellulose (A and B) and Ni^{2+}-Sepharose (C and D). In A and B, SDS-PAGE and Western blotting, respectively, of DEAE-cellulose eluates are shown: the solubilized fraction (lane 1), the passed-through fraction (lane 2), DEAE-cellulose eluate combined fractions #1-10 (lane 3), fractions #11-16 (lane 4), fractions #17-20 (lane 5), fractions #21-26 (lane 6), fractions #27-34 (lane 7), and fractions #35-42 (lane 8). Lanes 9-14 show each fraction of #21-26, respectively, which were subjected to Ni^{2+}-Sepharose affinity chromatography. In C and D, SDS-PAGE and Western blotting, respectively, of Ni^{2+}-Sepharose eluates are shown: DEAE cellulose fractions #21-26 (S), the passed-through fractions (P), fractions #1-3 washed with PBS containing 40 mM imidazole (Wash: lanes 1-3), fractions #1-5 eluted by PBS containing 400 mM imidazole (Elute: lane 1-5). Protein bands were stained with CBB (A and C). Western blotting with anti His-tagged mouse monoclonal antibody followed by anti-mouse IgG-HRP was carried out to stain the 1G11 scFv protein (B and D).

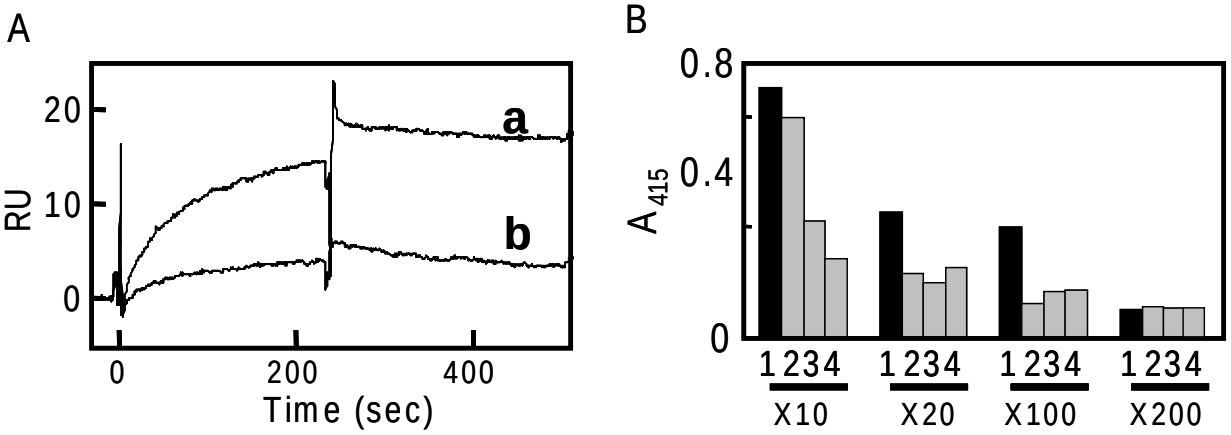

Figure 4. Tn-antigen binding activity of the purified 1G11 scFv protein. A: SPR analysis of the DEAE-cellulose eluates (a) and solubilized fractions (b). Binding to T-antigen HSA is shown. B: ELISA was carried out to determine T-antigen binding activity of the affinity purified 1G11 scFv (42.5 μg/mL). Shown are dose dependencies of binding activities against T-antigen E6-BDB (1: ■), Tn-antigen E6 BDB (GalNAcα-) (2), E6-BDB (3) and BSA (4).

DPPE as an antigen (*14*), only 15 out of 672 clones (2.2%) with intact scFvs were obtained. In addition, unlike isolation of M3 scFvs in which 15 clones had all different sequences, three and two identical clones were obtained for Group 1 and 4 phages, respectively, when screened by T-antigen E6-BDB. Such a concentration of binding to an antigen during panning is a good indication of possible positive clones being screened. The different outcomes from two studies may be due to the nature of their different carbohydrate moieties or the difference in the design of neoglycolipids used, namely the presence of E6 in the case of screening T-antigen scFvs. In contrast, panning and screening of phages with Tn-antigen E6-BDB did not yield any positive

clones although 928 clones were screened by ELISA. These results clearly suggested that while the binding of T-antigen consisting of disaccharide to antibodies can be achieved, Tn-antigen, which is the monosaccharide, is not big enough to stably bind to the binding domains of antibodies.

Characterization of phages isolated with T-antigen E6-BDB revealed that there are largely four different scFvs, Group 1-4. Group 4 consisting of two identical clones, 1G11 and 1H11, apparently showed high affinity and specificity for T-antigen when analyzed as a phage antibody, but are incomplete forms of scFvs, consisting of VH domain and only 30 amino acids of VL domain consisting of FWR1 and FWR4 and lacking most of the VL domain, CDR1, FWR2, CDR2, FWR3, and CDR3. Although it was obtained as an artifact in this case, since these clones were derived from a phage library with a large repertoire of natural and engineered human scFv genes, it resembles heavy-chain antibodies, so-called camelids (*19,20*). Camelids possess a functional class of antibodies devoid of light chains. The Group 4 clones may provide us with experimental models to test the role of VL domains in the formation of antigen-binding sites.

Besides binding to T-antigen, those phage antibodies except the Group 4 clone showed crossreactivity to Tn-antigen and LNFPIII. It is ultimately required to express and purify scFv proteins before their binding affinities and specificities can be evaluated. As a first step, 1G11 scFv (Group 4) protein was purified to near homogeneity by two column chromatographies from soluble fractions of *E. coli* expressing the scFv protein. The purified 1G11 scFv bound to T-antigen with greater affinity than Tn-antigen, but its binding activity seemed to be much lower than expected. This was also supported by SPR analyses which did not yield sensorgrams comparable to that of DEAE-cellulose eluates (Figure 4A-a). These results indicated that the purified 1G11 may be inactivated as a result of exposure to 2 M urea during Ni^{2+}-Sepharose affinity chromatography and that refolding had not been completed during elution with 400 mM imidazole/PBS. It is obvious that we need to improve procedures for expression and purification of the fractions for further characterization of scFv proteins and determination of affinity constants. Expression of Group 1-4 scFv proteins in *E. coli* at high levels and subsequent purification are now in progress, and in fact, a binding constant (KD) of 8×10^{-7} M for T-antigen HSA has been obtained for one of the scFv proteins purified (manuscript in preparation). Such production and purification of anti-T-antigen scFv proteins would provide the basis for developing antibody-based cancer therapeutics.

Acknowledgments

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Original Article

Localization of *c-mos* mRNA around the animal pole in the zebrafish oocyte with Zor-1/Zorba

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Summary **In oocytes, many maternally supplied products are stored, and these products play important roles in cell cycle regulation and early development. Mos protein, which is coded on the *c-mos* gene, promotes oocyte maturation and is involved in MAP-kinase signaling pathway. In *Xenopus*, maternally supplied *c-mos* mRNA undergoes poly(A) addition, and translational activation *via* CPE (cytoplasmic polyadenylation element) and CPEB (CPE-binding protein). The elongated poly(A) is shortened and the *c-mos* mRNA is degraded during early embryogenesis *via* EDEN (embryo deadenylation element) and EDEN-BP (EDEN-binding protein).**

We cloned the full-length zebrafish *c-mos* gene, which is conserved at the protein coding region in vertebrates. *c-mos* mRNA has two putative CPE sequences in its 3'UTR, which binds to zebrafish CPEB homologous protein, Zor-1. We could not observe EDEN sequence, and could not detect interaction between *c-mos* mRNA and zebrafish EDEN-BP homologous protein, Brul, even though immuno precipitation and RT-PCR experiments suggested that *c-mos* mRNA interacts with Zor-1 *in vivo*. Interestingly, we found *c-mos* mRNA is located in the animal cortex of zebrafish oocyte, where Zor-1 protein exists. Taken together, these results suggest that the animal cortex is the central core of oocyte maturation in zebrafish.

Keywords: CPEB, *c-mos*, CELF/Bruno, RNA localization, oocyte maturation

1. Introduction

In vertebrates, the cell cycle of an oocyte is arrested at the first prophase of meiosis. Oocyte maturation is initiated by maturation promoting factor (MPF) and is stopped in Metaphase II by cytostatic factor (CSF) before fertilization (*1*,*2*). The activated MPF promotes the cell cycle to overcome the Metaphase I check point. It causes germinal vehicle break down (GVBD) (*1*,*2*). Activities of CSF keep the cell cycle in Metaphase II of meiosis (*1*,*2*). These activities are regulated by the quantitative level of these components. MPF is composed of cdc2 and cyclin B. The translation of *cyclin B* mRNA is activated and cyclin B protein begins to increase from the initiation of the GVBD (*3*,*4*). Mos protein, a component of the CSF which acts as a serine/threonine kinase, increases greatly during oocyte maturation, and acts as MAPKKK (mitogen-activated protein kinase kinase kinase) (*3*,*4*). Mos protein and cyclin B protein synthesis are activated at the translation level of these mRNAs during oocyte maturation (*5*,*6*). In *Xenopus*, *cyclin B*1 and *c-mos* mRNAs are maternally supplied and stored with short poly(A) tails (20~50 nt). After the stimulation of oocyte maturation, poly(A) tails of these maternally supplied mRNAs are elongated to 150~200 nt and their translation is activated. After fertilization, these elongated poly(A) tails are shortened, and these mRNAs are degraded rapidly (*7*,*8*).

The polyadenylation and translational activation of *cyclin B1* and *c-mos* mRNA are controlled by *cis*-elements, which are CPEs (cytoplasmic polyadenylation elements, U5AU, U4A2U) and poly(A) addition signals (AWUAAA) of the 3' untranslationed region (UTR)

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in mouse and *Xenopus*. In this process, CPEB (CPE binding protein) binds to the CPE with Maskin protein (*9*). The binding between CPEB and CPEs depends on two RRMs (RNA recognition motifs) and a Zinc finger motif (ZnF) (*10*). The phosophorylation of CPEB raises a conformation arrangement and is forced to assemble with PAP (poly(A) polymerase) *via* binding to CPSF (cleavage and polyadenylation specific factor), which binds to the poly(A) addition signal (*11*). Since PAP carries out the polyadenylation of CPE containing mRNAs, elongated poly(A) containing mRNAs are produced. It is known that these mRNAs form circular structures, based on the interaction between elongated poly(A) and the eIF4F at its 5' cap structure, *via* binding to PABP (poly(A) binding protein). Then, this structure promotes recycling of 40S ribosomal complex, and activates the translation of its mRNA. Deadenylation of poly(A) tails after fertilization has been reported in *Xenopus c-mos* and *Eg5* mRNA. The deadenylation of these mRNAs depends on embryo deadenylation elements (EDENs), which specifically bind to EDEN-binding protein (EDEN-BP) (*12*). EDEN-BP is an RNA recognition motif (RRM) type RNA-binding protein, belonging to CELF/Bruno-like family. Bruno, one of the members of CELF/Bruno-like family, acts as the translational repressor of *oskar* and *gurken* mRNAs in *Drosophila* oocytes (*13*). Another family member, CUGBP1, plays a very important role in myotonic dystrophy *via* splicing regulation (*14*). Besides translational regulators, CPEB and EDEB-BP, it is clear that microRNAs control the translational levels of the target mRNAs in recent studies. For example, miR-430 mediates deadenylation and clearance of maternal mRNA in early embryogenesis in zebrafish (*15*).

In zebrafish oocytes, some maternally supplied products are localized at the cortex of the animal pole or vegetal pole. *zor-1/zorba* mRNA, which is a *CPEB* homologoue in zebrafish, and *cyclin B* and *pabp* mRNAs are also localized at the cortex of the animal pole in grown oocytes (stage III) (*16,17*). Zor-1 protein expression corresponds to that of its mRNA localized at the animal cortex (*16*). Previously, we reported that *zdazl* and *brul* mRNAs are localized at the cortex of the vegetal pole in zebrafish oocytes (*18,19*). Brul protein is a homologous protein of *Xenopus* EDEN-BP and human CUGBP1, and can specifically bind to the EDEN sequence (*20*). *zdazl* mRNA, *brul* mRNA and Brul protein are transported to the distal ends of the cleavage furrows of the 4-cell stage embryo, where the germ plasma is formed (*21,22*). In addition, *vasa*, *nanos1*, *dnd*, and *askopos* mRNAs were also detected in the cleavage furrows (*23-26*).

Although the zebrafish *c-mos* gene has already been reported (*27-29*), we independently cloned a *c-mos* homologous gene in zebrafish. Zebrafish *c-mos* gene encodes the Ser/Thr kinase domain and has CPE-related sequences in its 3'UTR without the EDEN sequence. The *c-mos* mRNA is localized around the animal pole in zebrafish oocytes. Furthermore, our results suggest that *c-mos* mRNA binds to the Zor-1 protein *in vitro* and *in vivo*, but does not bind to a vegetal localized factor, Brul protein. Therefore, the embryonic deadenylation regulated by EDEN-BP may not be essential for *c-mos* mRNA, but the cytoplasmic polyadenylation of *c-mos* mRNA is one of the basic mechanisms in vertebrates.

2. Materials and Methods

2.1. *RT-PCR and cDNA screening*

Two degenerate primers, MOS/DS (CCAGAATTCTTY TGGGCNGARYTNAAY) and MOS/DA (TCTGTCGA CATYTGCCANARNGTDAT), were prepared based on *c-mos* genes in vertebrates. The zebrafish *c-mos* cDNA fragment was amplified by RT-PCR with MOS/DS and MOS/DA primers from total ovarian RNAs (TaKaRa). The digoxigenin (DIG)-labeled fragment was amplified with CM/PS primer (ATTAAGCTTCAAAACATTG TGCGCGTG) and CM/PA primer (CAAGGATCCGC CAAAAGAATAAACGTC). A zebrafish adult cDNA library (a gift from Dr. Grunwald) in the λZAPII vector was screened under high stringency conditions with the DIG-labeled fragment. Excision of positive phagemid sequences was carried out according to the description supplied by Stratagene. Three independent clones contained the zebrafish *c-mos* cDNA. The sequence of *c-mos* gene has been deposited in the DDBJ (#AB032727).

2.2. *Northern blot*

Zebrafish (*Danio rerio*) were maintained at 28.5°C on a 14 h light/10 h dark cycle. Gonads were manually isolated from mature adult fish. Total RNA was purified from embryos, whole females and males, the dissected ovaries and testes, and the remaining bodies after dissection. For northern blotting of adult and adult tissues, 10 μg of total RNA were electrophoresed as shown in Figure 2A and Figure 2B *lane* 1. For northern blotting of embryonic stages, 20 μg of total RNA were electrophoresed as shown in Figure 2B *lanes* 2-5. The DIG-labeled cDNA fragments were used as probes. For detection, CSPD (Tropix) was used as a chemiluminescent substrate.

2.3. *In situ hybridization*

Whole mount and sections of *in situ* hybridization was performed essentially as described (*19*). The full-length of the zebrafish *c-mos* gene was used as a probe. For *in situ* hybridization of ovarian sections, specimens were embedded in paraffin and cut at thickness of 9 μm. Stages of oocytes were classified as described (*16*).

2.4. *Preparation of GST fusion protein*

Portions of Zor-1 coding sequence were amplified by PCR, and subcloned into pGEX 6p-1 (Amersham). The Zor-1 primer sets are as follows: GS1 (CAGGAATTC GCCATGGCGTTTTCTCTGA) and GAS1 (GCAGTC GACCACATGGACATCCAGG CTC) for pGST-Zor-1, GS1 and GAS2 (GCAGTCGACTAGGCATCCTCCA AGTATG GATC) for pGST-ΔZnF. Each expression plasmid was transformed in *E. coli* DH5α. GST fusion proteins were induced with 0.1 mM IPTG. GST-Zor-1 and GST-ΔZnF proteins were affinity purified and dialyzed with binding buffer (10 mM Hepes-KOH (pH 7.7), 100 mM KCl, 5% glycerol, 1 mM $MgCl_2$, 1 mM DTT, 0.2 mM PAMSF, 5 μg/mL Pepstatin A). Concentrations of these proteins were calibrated using CBBR-250 (Bio-Rad) and densitometry was performed after SDS-PAGE (MacBAS, Fuji).

2.5. *UV-crosslinking experiments*

Annealed oligo nucleotides corresponding to the *Xenopus* B4-type CPE and the mutant CPE (*7*) were cloned into pSP64 (Promega). Portions of annealed oligonucleotides of the zebrafish *c-mos* 3'UTR; zm-CPE1 (AAATTTTTTATGCAAAATGTTTAATTAA AT GT), zm-CPE2 (AAATGTTCGTGTTTTTGTTT TATTGTGAAGCT) and zm-nc (ATA ACAATTGTT TAATATTGTAAATGTTCGTGT), were cloned into pSP73 (Promega). *In vitro* transcription was carried out as described previously, in the presence of [α-^{32}P] UTP (*30*). UV-crosslinking experiments were performed essentially the same as described (*31*). RNA probes were incubated with the fusion proteins in binding buffer containing 0.5 μg/μL of yeast tRNA at 23°C for 20 min. After UV-irradiation and RNaseA-treatment, the reaction mixture was applied onto SDS-PAGE. Images of binding products were analyzed using a BAS2000 Image Analyzer (Fuji).

2.6. *IP- RT-PCR*

Whole ovaries from adults were harvested and homogenized in ice-cold extract lysis buffer (15 mM Hepes-KOH (pH 7.9), 50 mM KCl, 6.25 mM $MgCl_2$, 5% glycerol, 0.1% NP-40, 0.1% Triton X-100, 1 mM DTT, 0.2 mM PASMF, 1 mM EDTA). After pelleting yolk and debris, the supernatants were combined with RNase inhibitor (Promega).

The anti-Zor-1/Zorba antibody and the pre-immune serum were a gift from Dr. Bally-Cuif (*16*). The anti-Brul was prepared essentially as described previously (*21*). The pre-immune serum, Zor-1- and Brul-antibody were each separately mixed with Protein G Agarose (GE). 200 μg of oocyte extract was added to each of above mentioned solutions. Gels were washed five times with washing buffer (10 mM Hepes-KOH (pH 7.9), 400 mM KCl, 5% glycerol, 1 mM DTT, 0.2 mM PAMSF). Immunoprecipitated RNA was purified by phenol/chloroform extraction followed by ethanol precipitation. We carried out the reverse transcription reaction and the polymerase chain reaction (PCR) with an RT-PCR kit (TaKaRa) and specific primers of zebrafish *c-mos*; CM/PS and CM/PA, eIF 4A; eIF4A5' (TAAGGATCCGATTTACGAC GTGTACCG) and eIF4A3' (ATTGAATTCCAAACGTCTGTGGAG ATC) and dazl mRNA; dazl5' (GTAGGATCCATG GTTCAGGGGGTTCAG) and dazl3' (GTCCTCGA GCTACATAAGGGTTAGCAAAG). The RT-PCR products were electrophoresed in 6% native polyacrylamide gels. After staining with SYBR Green I (Molecular Probe), the images of the products were analyzed using an FM-BIO II (HITACHI) bioimager.

3. Results

3.1. *Cloning of zebrafish c-mos gene*

We designed degenerate primers that contain well conserved regions of *c-mos* gene from several species. To isolate the zebrafish *c-mos* homologus gene, we carried out RT-PCR experiments and cDNA screening from the zebrafish whole adult cDNA library. Three independent clones were obtained and sequenced (Figure 1). Three clones are essentially identical, and are similar to *Xenopus c-mos* gene. Northern blot analysis showed that the *c-mos* mRNA was highly expressed as a single approximately 1.3 kb product (Figure 2A). This size matched the *c-mos* cDNA which we cloned. This suggested that the *c-mos* cDNA contained almost the full length of the zebrafish *c-mos* gene. The zebrafish *c-mos* gene encodes the Ser/Thr kinase domain and contains the 50 nt of the poly(A) tail, the polyadenylation signal (AUUAAA) and two putative CPE sequences (Figure 1). The polyadenylation signal and putative CPEs are located within 50 nt of the 3'UTR. We could not detect EDEN-conserved sequence in the zebrafish *c-mos* gene.

3.2. *Expression and localization pattern of c-mos*

To examine expression patterns of *c-mos* gene, northern blot analysis was performed in adult fish at first. The *c-mos* mRNA was detected in the RNAs of females and ovaries, but was not detected in the RNAs of somatic organs of female or male fish (Figure 2A). This result shows that the *c-mos* mRNA was highly and specifically expressed in ovaries.

To examine when the *c-mos* gene was expressed in zebrafish oogenesis, we carried out whole mount *in situ* hybridization. The *c-mos* mRNA was expressed during early oogenesis, in stage Ia and Ib oocytes (data not shown). Before stage III, the *c-mos* transcripts were detected throughout the oocyte (Figure 3A). Interestingly, *c-mos* mRNA was localized in one part

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AAATTAGCGACCAGCTCTCCGATTTCACAGTTATCATGCGCCATGCCCTCACCAATCCCC      60
                                                  M  P  S  P  I  P
GTCACCCGACTTTTGCCAAAGGATTTCGGCCTCGAGTTTGGCGCATGCAGCAGCCCGCTG     120
V  T  R  L  L  P  K  D  F  G  L  E  F  G  A  C  S  S  P  L
ACCAAAACTGCCAGTGGATCTACCCTGCGCGTGCCCACAAACAAGTTTCATGGTAAAGTC     180
T  K  T  A  S  G  S  T  L  R  V  P  T  N  K  F  H  G  K  V
GCACACAGGCTCTGGTCCTCCGTGATCCACTGGCGCGAGCTGCAGGCTCTGGAGCCCATA     240
A  H  R  L  W  S  S  V  I  H  W  R  E  L  Q  A  L  E  P  I
GGCAGCGGTGGATTCGGTACGGTGTTCAGAGGCACATACTTCGGCGAGACTGTCGCTGTG     300
G  S  G  G  F  G  T  V  F  R  G  T  Y  F  G  E  T  V  A  V
AAAAAGGTCAAGTGTGTGAAAAACAAACTGGCATCGAGGCAAAGTTTCTGGGCGGAACTC     360
K  K  V  K  C  V  K  N  K  L  A  S  R  Q  S  F  W  A  E  L
AACGCCGCGCACCTGCACCATCAAAACATTGTGCGCGTGCTCGCGGCCACCACGTGCACT     420
N  A  A  H  L  H  H  Q  N  I  V  R  V  L  A  A  T  T  C  T
CCTGCGCATCTCAACACCAAAGACAACATCGGGACGATCGTAATGGAGTTCGCAGGCAAT     480
P  A  H  L  N  T  K  D  N  I  G  T  I  V  M  E  F  A  G  N
ATAAATCTACAGAAGCTCATTTATGGGCTCACAGACTTGCTTCCTGTGGAGAAGTGTATA     540
I  N  L  Q  K  L  I  Y  G  L  T  D  L  L  P  V  E  K  C  I
AAGTATTCAATAGACATCGCGCGCGCCCTCCAGCACCTGCACGCGCACGGCGTAGTGCAC     600
K  Y  S  I  D  I  A  R  A  L  Q  H  L  H  A  H  G  V  V  H
CTGGATTTAAAACCAGCCAATGTCTTGTTGTCAGAACAGGGTGTTTGTAAAATCGCAGAT     660
L  D  L  K  P  A  N  V  L  L  S  E  Q  G  V  C  K  I  A  D
TTTGGGTGCTCGTTTAAAATATCCAGCACAAGTGACACCGTGACGCACATGAATGAAATC     720
F  G  C  S  F  K  I  S  S  T  S  D  T  V  T  H  M  N  E  I
GGCGGCACGTTTACGCACCGGGCGCCCGAGCTGCTGAAAGGTGAGGAAGTGTCGCCGCGC     780
G  G  T  F  T  H  R  A  P  E  L  L  K  G  E  E  V  S  P  R
GTGGACGTTTATTCTTTTGGCATAACGTTGTGGCAGCTGCTCACCCGAGAGCCGCCCTAT     840
V  D  V  Y  S  F  G  I  T  L  W  Q  L  L  T  R  E  P  P  Y
GAGGGAGACAGACAGTATATCCTGTACGCTGTTGTGGGGTATAACCTGCGCCCTTTGACC     900
E  G  D  R  Q  Y  I  L  Y  A  V  V  G  Y  N  L  R  P  L  T
AGCAGGAATGTTTTTACCCAGTTTTTTATTGGACAGAATTGTCAAAAACTGATCAGCCGG     960
S  R  N  V  F  T  Q  F  F  I  G  Q  N  C  Q  K  L  I  S  R
TGTTGGGACGGCGACCCCAGCATCCGACCGACCGCAGATAAGTTTGTCGACGAACTTTCA    1020
C  W  D  G  D  P  S  I  R  P  T  A  D  K  F  V  D  E  L  S
GTTTTACTGTAAAATTGAATCTCCGCGTTTGCAAAAAAAAAAAAAAAAAAAAATCTTCAAT    1080
V  L  L  .
GAAACGTTTTTTTATAACAATTGTTTAATATTGTAAATGTTCGTGTTTTTGTTTTATTGT    1140

GAAGCTAAATTTTTTTATGCAAAAATGTTTAATTAAATGTTTCCTTGTGTTTAAAAAAAAA    1200

AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA                       1241
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Figure 1. Nucleotide and amino acid sequences of the zebrafish *c-mos* gene. The nucleotide sequence is indicated in black. The amino acid sequence is indicated with color and shows the open reading frame. The underlined section shows annealing positions of the degenerate primers. The yellow highlights indicate putative CPE (cytoplasmic polyadenylation element) sequences. The orange highlight indicates the polyadenylation signal.

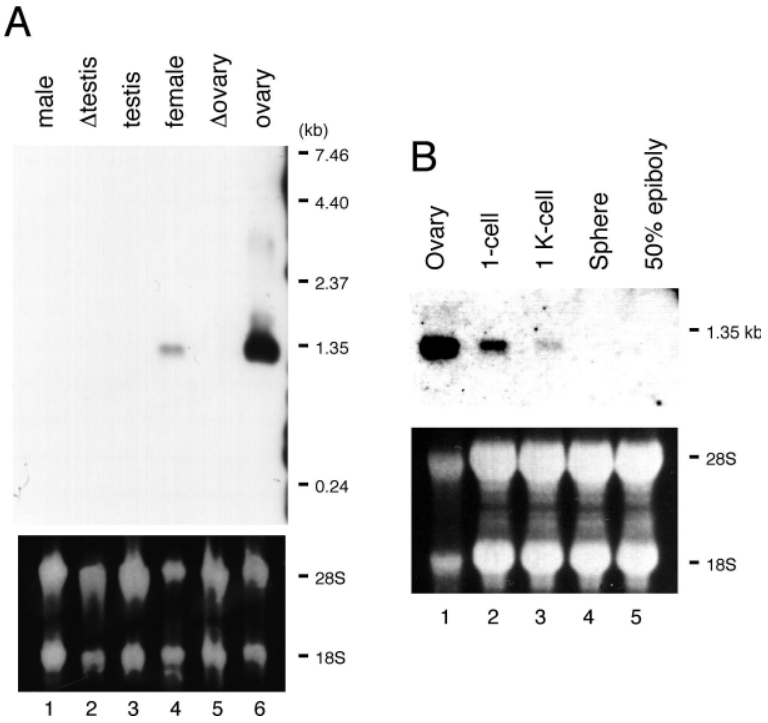

Figure 2. Northern blot analysis for zebrafish *c-mos* mRNA. The upper panels show *c-mos* mRNA. As a control, the lower panels show ribosomal RNAs. The RNA size markers are indicated on the right side of the panels. (A) The expression analysis of *c-mos* mRNA in adult zebrafish. 10 μg of total RNA from adult male, Δtestis, testis, adult female fish, Δovary and ovary are applied in each lane. Δtestis and Δovary indicate somatic tissues from adult fish, not including testis or ovary, respectively. (B) The expression analysis during early embryogenesis. Ten μg of total RNA from the ovary and 20 μg of total RNA from the embryo are applied to each lane.

of stage III oocytes (Figures 3B and C). To clarify where *c-mos* mRNA was localized, we performed *in situ* hybridization on ovary sections. The micropyle, indicated as the future animal pole, was observed in oocytes after stage III and *c-mos* mRNA was detected in the cortex around the micropyle (Figure 3D). These results show that the zebrafish *c-mos* mRNA is localized in the cortex of the future animal pole in oocytes after stage III. In a previous study, Bally-Cuif *et al*. (*16*) reported that *zor-1/zorba* mRNA and Zor-1/Zorba protein was localized around the future animal pole in zebrafish oocytes. To investigate whether the region of *c-mos* mRNA localization is the same as that of *zor-1* mRNA, we performed *in situ* hybridization on continuous sections. We observed that the *c-mos* and *zor-1* mRNAs were co-localized approximately in the animal cortex of stage III oocytes (Figures 3D and E). These results show that *c-mos* mRNA is one of the animal pole localized factors in zebrafish oocytes (*16*).

After fertilization, the *c-mos* mRNA was detectable in the blastomere of 1-cell stage embryo (Figure 3F). This mRNA completely disappeared between 1k-cell stage and sphere stage (Figures 3G and H). Northern blot analysis showed that the *c-mos* mRNA decreased in the maternal stage embryos, and was not observed in the RNA of sphere stage embryo (Figures 2B). From

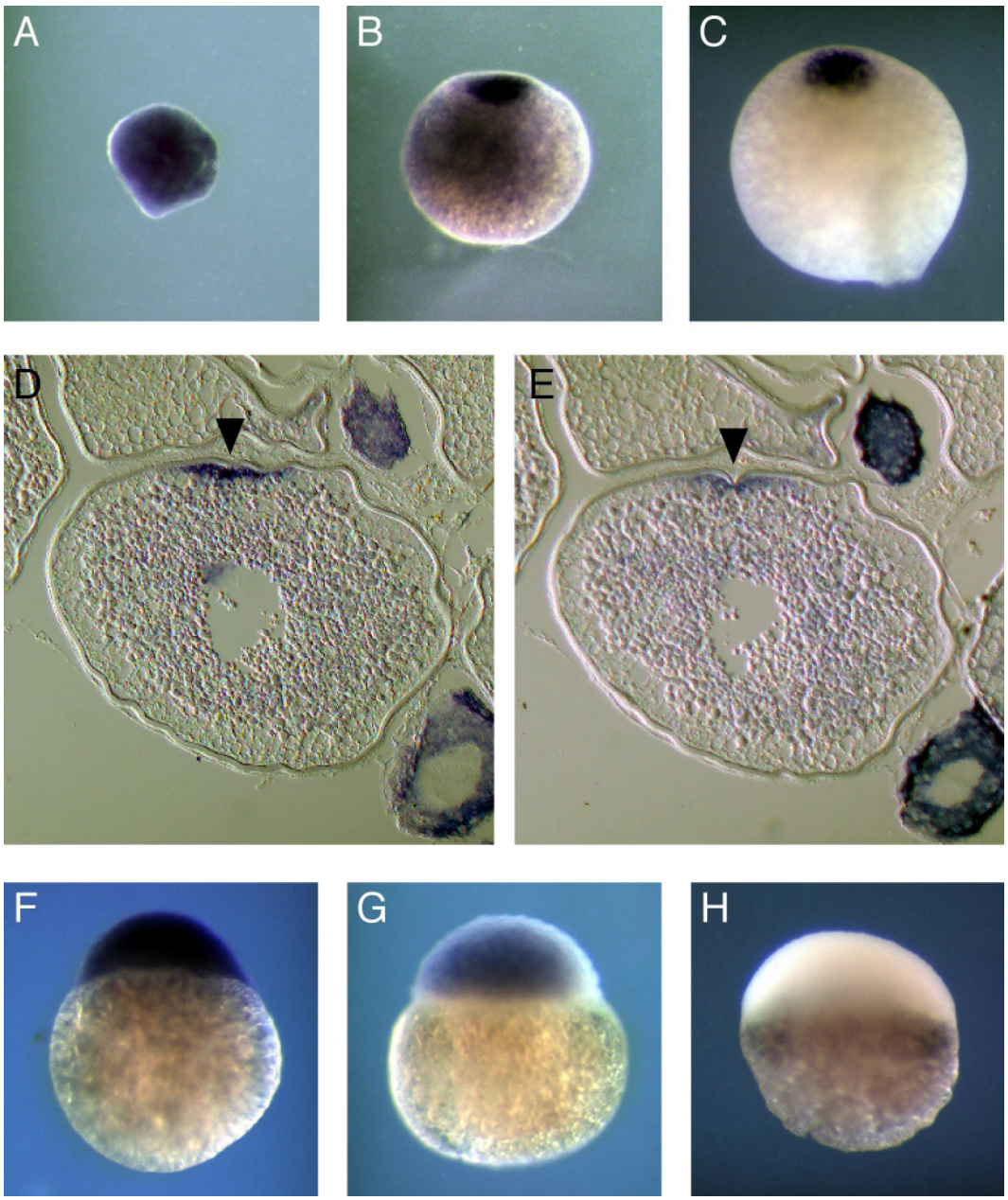

Figure 3. *In situ* hybridization of *c-mos* mRNA in zebrafish oogenesis and early embryogenesis. (A-C) whole mount *in situ* hybridization of *c-mos* mRNA during oogenesis. The expression patterns of *c-mos* mRNAs at stage II oocyte (A), early stage III oocyte (B) and stage III oocyte (C) are shown. The serial vertical sections are used for *in situ* hybridization to analyze the expression of *c-mos* mRNA (D) and *zor-1* mRNA (E) as a control. The arrowheads indicate the micropyles, which indicate the animal pole of zebrafish oocytes. (F-H) whole mount *in situ* hybridization of *c-mos* mRNA during embryogenesis. The expression patterns of *c-mos* mRNAs in the 1-cell stage embryo (F), the 1k-cell stage embryo (G) and the sphere stage embryo (H) are shown.

these results, it is suggested that *c-mos* mRNA was rapidly destroyed during early embryogenesis, like *Xenopus c-mos* and *cyclin B1* mRNAs.

3.3. *Binding between the CPEs of c-mos mRNA and Zor-1 protein in vitro*

Zor-1 protein, which is encoded by the animal pole localized *zor-1* mRNA, was localized around the animal pole of oocytes along with *cyclin B*, *pabp*, *Vg1* and *c-mos* mRNAs (*16*, Figure 3D). Since the *zor-1* gene is a highly conserved CPEB homologus gene in zebrafish (*16*), we analyzed whether the zebrafish Zor-1 specifically bound to the CPE using UV-crosslinking experiments. We found that the Zor-1 bound to the CPE of *Xenopus cyclin B1* mRNA, but did not bind to a mutated RNA of the CPE (Figure 3C, *lanes* 1 and 2). This showed that Zor-1 bound to the CPE specifically. In *Xenopus*, it has been shown that the ZnF of CPEB is important for the CPE-binding in a manner dependent on Zinc ion (*10*). The lack of a ZnF in Zor-1 disrupted the CPE-binding (Figures 4B and 4C). The lack of Zn^{2+}

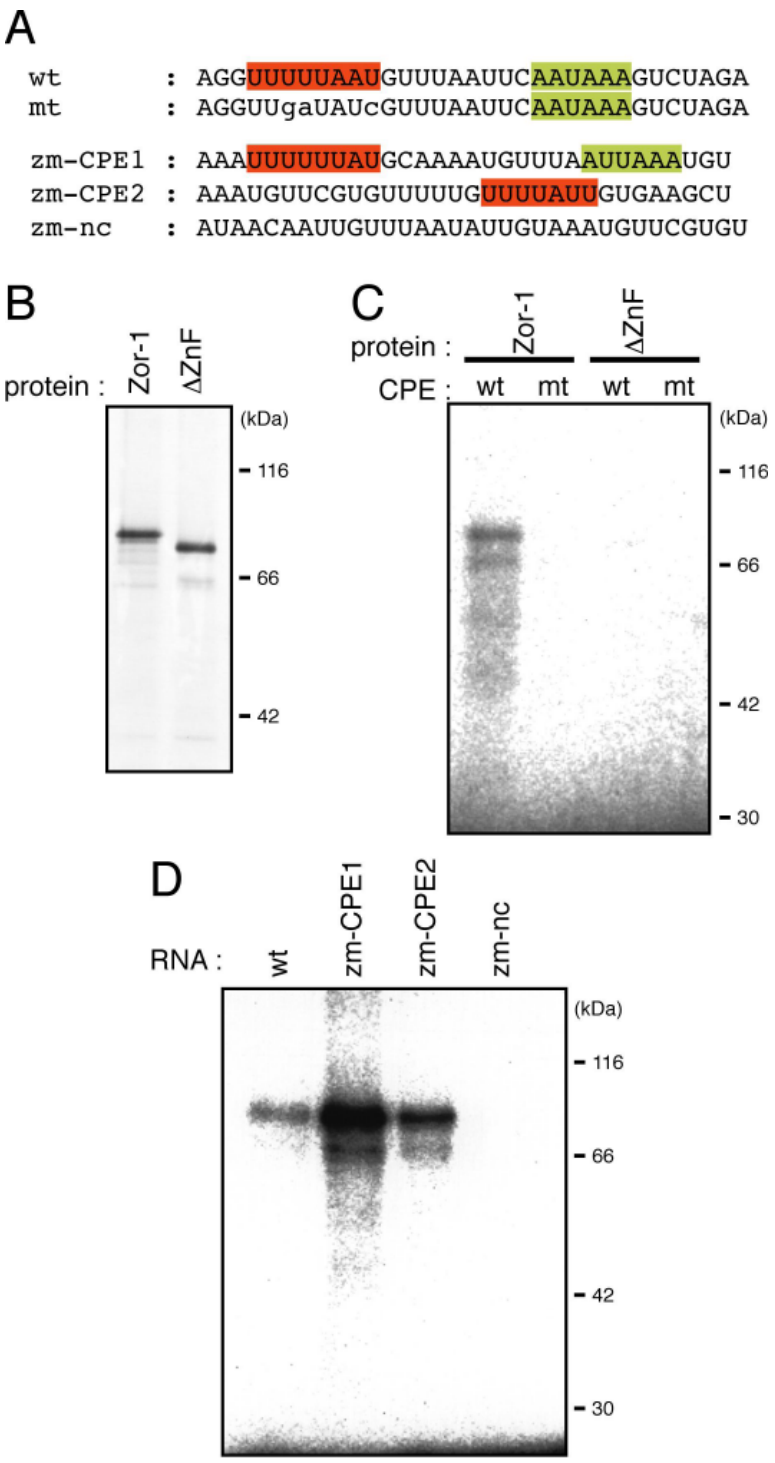

Figure 4. UV-crosslinking experiments between zebrafish *c-mos* and Zor-1 protein. ^{32}P labeled RNA sequences are shown (A). The first line is the CPE sequence of *Xenopus cyclin B1*, and its mutant is in the second line. The other lines indicate two putative CPE sequences, and a control sequence in zebrafish *c-mos* mRNA. As described in Figure 1, the CPE sequences and polyadenylational signals are highlighted in orange (CPE) and yellow (poly(A) signal). Zebrafish CPE binding protein, Zor-1, and its deletion mutant proteins of zinc finger domain (ΔZnF) are prepared as GST-fusion proteins, and analyzed in SDS PAGE with CYBR staining (B). UV-crosslinking experiments were carried out using Zor-1 proteins with the CPE RNA of *cyclin B1* (C) and with the putative CPE sequences of *c-mos* (D).

ion in the binding reaction also reduced the binding activity of Zor-1 with the CPE (data not shown). These results mean that the similarities between the zebrafish Zor-1 and the *Xenopus* CPEB are not only amino acid identities but also CPE-binding.

As we described above, zebrafish *c-mos* mRNA has two putative CPE sequences (Figure 1). Therefore, we checked whether these CPE sequences were bound to Zor-1 protein. We found that Zor-1 protein bound strongly to the CPE1 and CPE2 sequences, but not to the non-CPE sequence in zebrafish *c-mos* mRNA (Figure 4D). The binding of Zor-1 with CPE1 or CPE2 of zebrafish *c-mos* mRNA seems to be much stronger than that of Zor-1 with the *Xenopus* CPE.

3.4. *The interaction between the c-mos mRNA and Zor-1 protein in vivo*

We detected the binding activity of Zor-1 protein to the CPEs of zebrafish *c-mos in vitro*. Our results and previous studies have shown that maternally supplied *c-mos* mRNA and Zor-1 protein exist in the animal cortex of zebrafish oocytes (*16*). Therefore, we tested whether Zor-1 protein interacted with *c-mos* mRNA *in vivo* using IP-RT-PCR (immuno precipitation and RT-PCR). The band of *c-mos* mRNA was detected in co-precipitated RNA by Zor-1, but not in RNA of the pre-immune serum (Figure 5). We tested *eIF4A* mRNA as a negative control. Actually, we could not detect any PCR products from *eIF4A* mRNA. These results suggested that *c-mos* mRNA and Zor-1 protein were associated in zebrafish oocytes.

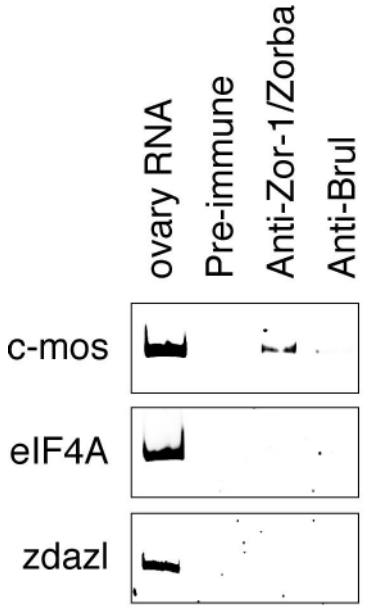

Figure 5. Immunoprecipitation and RT-PCR experiment. Anti-Zor-1/Zorba, anti-Brul and pre-immune serums were each separately mixed with protein G resin and prepared as Antibody conjugated columns. Oocyte extracts were applied to these columns. The RNAs co-precipitated with either Zor-1 or Brul protein, were purified and used for the RT-PCR. The left lane shows the PCR products from *c-mos*, *dazl* and *eIF4A* mRNAs, as controls which were amplified from 200 µg of oocyte extracts. The PCR products of co-purified RNA were analyzed in 6% native acrylamide gel, stained with SYBR Green I, and imaged with the FM BIO II (Hitachi).

Although *Xenopus c-mos* mRNA has the EDEN sequence, which can be bound to EDEN-BP, zebrafish *c-mos* mRNA doesn't have any EDEN-like sequence. Furthermore, *brul* mRNA, zebrafish EDEN-BP homologous gene transcript, is mainly localized at the vegetal cortex in the oocyte. Brul protein is detected in the cleavage furrows in 4-cell stage embryo (*22*), but the localization of Brul protein in oocytes is unclear. Indeed, this was consistent with our result that we could not detect any interaction between *c-mos* mRNA and Brul protein in the IP RT-PCR assay (Figure 5).

4. Discussion

We have identified the full-length of *c-mos* gene whose transcript was specifically expressed in ovary. We found that *c-mos* mRNA, which was detected throughout oocyte stage Ia and Ib, was localized in the animal cortex in the oocyte after stage III. Since *c-mos* mRNA conserved two putative CPE sequences in its 3'UTR, we assume that it is involved in the cytoplasmic polyadenylation event. Actually, we showed the animal pole localized CPEB homologue, Zor-1 protein interacting with *c-mos* mRNA *in vivo* and *in vitro*. These results suggested that the spatial regulation of translational control was very closely related to oocyte maturation in zebrafish.

After fertilization, *c-mos* mRNA was completely degraded during early embryogenesis. However, the zebrafish *c-mos* gene lacks any EDEN-like sequence (Figure 1). No interaction between *c-mos* mRNA and Brul protein was detected by the IP-RT-PCR experiment (Figure 5). Furthermore, *c-mos* mRNA is localized at the animal cortex of oocytes, though *brul* mRNA is mainly localized at the vegetal cortex (*19*). These results suggest that there was no remaining mechanism for the embryonic deadenylation of *c-mos* mRNA, in contradiction to reported *Xenopus* results (*12*). However, Northern blot analysis showed that maternally supplied *c-mos* mRNA was rapidly degraded in early embryogenesis (Figure 2B). Therefore, we need to consider other mechanisms for the degradation. It became clear that microRNAs are strongly affected by RNA metabolism and its translational repression. For instance, miR-430 plays a very important role in the degradation of maternally supplied mRNAs in somatic cells during early embryogenesis (*15*). There is so far no report that miRNAs regulate *c-mos* mRNA in vertebrates. Although, we preliminarily searched for the miR-430 binding site in 3'UTR of zebrafish *c-mos* mRNA, we could not detect any binding site.

On the other hand, zebrafish *c-mos* mRNA has two CPE sequences in its 3'UTR (Figure 1). We found that Zor-1 protein interacted with zebrafish *c-mos*

mRNA *in vivo* and *in vitro* (Figures 4D and 5). *c-mos* mRNA was localized at the animal cortex of zebrafish oocytes (Figure 3). Similarly, it was reported that *zor-1* mRNA was localized at the animal cortex (*16*). Taken together, it is likely that the machinery for cytoplasmic polyadenylation during oocyte maturation is conserved in zebrafish and *Xenopus*. Many of the factors, involved in the cytoplasmic polyadenylation event, are located at the animal cortex in zebrafish oocytes. We assume that the animal cortex is a central location where oocyte maturation, including mRNA metabolism of *c-mos* mRNA takes place. However, it remains to be clarified whether or not *c-mos* mRNA undergoes cytoplasmic polyadenylation during oocyte maturation in zebrafish. Recently, Zhan and Sheets reported that zebrafish *cyclin B1* mRNA undergoes cytoplasmic polyadenylation *via* the CPE sequence, and was translationally activated in oocyte maturation (*32*).

One of the vegetally localized mRNAs, *zdazl*, reached the vegetal cortex *via* the METRO-like pathway (*33*). Although *vasa* and *nanos1* mRNAs, which are localized in the cleavage furrow and are essential for germ cell formation are not localized at the vegetal cortex, these mRNAs temporally join the pathway (*33*). Another vegetally localized mRNA, *brul* mRNA, reached the vegetal cortex *via* a late pathway known in *Xenopus* Vg1 mRNA localization (*19,34*). These METRO-like pathway-related mRNAs and vegetally localized mRNAs are finally localized at the distal ends of cleavage furrows in the 4-cell stage embryo, where zebrafish germ plasmas are located (*21*). However, many of the animally localized products are involved in cell cycle regulation during oogenesis as well as early embryogenesis. Since maternally supplied products stored in the oocytes are essential for metabolism and development of the zebrafish embryos, the oocytes are much larger than usual somatic cells. It is reasonable for oocytes and eggs to carry out a cellular event in a specific area, such as in the animal cortex. However, it is unclear how the animally localized mRNAs are transported and anchored to the animal cortex in the zebrafish oocyte. It has been reported that the vegetally localized mRNAs have multiple localization signals (*33*). Since the 3'UTR of zebrafish *c-mos* mRNA is quite short (~150 nt), this mRNA may become a good model for analyzing the mechanisms of animal pole localization in the zebrafish oocyte.

Acknowledgments

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Original Article

Increased protein expression of p16 and cyclin D1 in squamous cell carcinoma tissues

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Summary **Abnormalities of Rb-pathway components are common in the formation of cancer. The immunostaining for cyclin D1 and p16 protein was applied on 1 mm serial tissue microarray (TMA) paraffin sections. Tissue microarray (TMAs) is potentially a good method to find the molecular features of the genes and expressions of them. The aim of this study was to evaluate the protein expressions of cyclin D1 and p16 genes in squamous cell carcinomas (SCCs) of skin and compare with the normal skin tissue. Twenty-five cases of cutaneous SCCs expressed cyclin D1 and p16 proteins. All SCCs samples on the slides showed positive protein expressions of cyclin D1 and p16 genes. Our findings suggested that the increased protein expressions of cyclin D1 and p16 genes might lead to aberrant expressions of these proteins in the affected tumor cells. This study demonstrated that cell cycle controlled deregulation and uncontrolled cell cycle progression might result in the carcinogenesis.**

Keywords: p16, cyclin D1, retinoblastoma pathway, tissue microarray, squmous cell carcinoma

1. Introduction

D-type cyclins are the first cyclins expressed in the G1 phase of the cell cycle. Cyclin D1 (CCND1, PRAD1, bcl-1), cyclin D2, and cyclin D3 are three closely related D-type cyclins which are expressed in a cell lineage manner in all proliferating cells. Binding of cyclin D1 with cyclin-dependent kinase(CDK) 4 and CDK6 phosphorylates and inactivates the retinoblastoma protein (pRb). Deregulation of the G1 cyclins and CDKs expression might cause loss of the cell cycle control leading to tumorigenesis (*1*).

The p16 gene product is encoded by the ARF-INK4 locus. The p16 protein is a specific inhibitor of CDK/cyclin D complexes which prevent the phosphorylation of the pRb protein (*2*). It can arrest the cells in G1 phase (*2*) and thus suppress cell proliferation (*1*). pRb, p16, and CDK/cyclin D are functionally linked together and the alterations of these cell cycle regulator components might have similar results. Moreover, changes of at least one of these regulators were found in nearly all human cancers (*2*).

Skin cancer is one of the most common human malignancies. The most common types of skin cancers include basal cell carcinoma (BCC), squamous cell carcinoma (SCC), and malignant melanoma (MM). Chronic exposure to UV irradiation from sunlight is believed to be the cause of the majority of human skin carcinoma types like cutaneous SCCs and BCCs (*3*).

SCC is currently the second most common type of non-melanoma skin cancer. SCCs of the skin are associated with the risk of metastasis. Typically, SCC is a painless skin lesion that develops on sun-exposed regions of the body (*4*). Most SCCs occur on skins that have exposed to the sun such as head and neck, forearms, back of the hands, upper part of the torso, and lower legs (*5*). It may also arise in regions with scars

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from old burns or vaccinations (*4*).

Many of the SCC lesions develop in precancerous patches called actinic keratoses. SCCs are more common in the old adult population rather than the young. Overall, chance of developing SCCs is about 7-11% in the population (*5*). Incidence of SCC of the skin is rising worldwide for decades. The most important environmental risk factor for this type of skin cancer is chronic exposure to sunlight which induces the DNA damage (*6*).

SCC has become an increasing trouble for the general population and it is particularly life threatening in immuno-suppressed patients. The face, head, neck, back, hand, and forearm are preferential sites of chronic UV damage (*7*). Regarding the alarming rates and disease outcomes, it is more important than ever to understand the regulatory molecular genetic alterations which result in the skin cancer progression and finding correlation of these changes with modified cellular functions (*3*).

Finding the molecular markers in the tissue provides more information which are not detected by routine histopathology (*8*). The aim of this study was to evaluate the protein expression status of the cyclin D1 and p16 genes in the skin SCC and compare their expression in the normal skin tissue. It illustrated the molecular alterations which lead to the cell cycle proliferation and uncontrolled cell cycle progression.

2. Materials and Methods

2.1. *Samples*

Twenty-five spot samples of formalin-fixed, paraffin embedded AccuMax® TMA slides were obtained from twenty-five different patients from Korea (CAT#: A216; LOT#: 122120310121, Location: 73 and 74) who were diagnosed as SCC and 4 spot samples of normal skin tissues. Size of each spot is 1.0 mm in diameter. Immunohistochemistry (IHC) was performed on the slides using commercially available monoclonal anti-cyclin D1 antibody obtained from Research Biolabs (Danvers, USA) and anti-p16 polyclonal antibody named anti-p16INK4a from Lab vision (Fremont, CA, USA). Immunohistochemical staining was conducted using DAKO Envision TM system + HRP DAB + Rb /Mo Kit (DAKO Co., Carpinteria, CA, USA) according to the manufacturer's instructions.

Briefly, the slides were dewaxed by heating on hot plate at 60ºC and then deparaffinised. The slides were heated in a microwave oven for 20 min in 10 mM citrate-Na (pH 6.0). After incubation with dual endogenous blocking enzyme for 10 min, sections were incubated with primary antibodies with dilution of 1:400 for anti-p16 and 1:300 for anti-cyclin D1 overnight at 4ºC. After washing by TBS, the slides were incubated with polymer in the kit for 30 min followed by an incubation with DAB+ substrate buffer for labelling for 10 min. After counterstaining with hematoxylin and mounting, the slides were viewed by light microscope (BX 51, New York, USA).

2.2. *Scoring system*

Semi-quantitative assay may improve standardization and reproducibility of the study in the laboratories as well as minimize the impact of many known inconsistencies (*9*). As the strength of reaction was variable, we graded the intensity of reaction on a numerical scale from + to +++, reflecting weak, moderate, and strong reactions. We also recorded the extent of reactivity in the target cell population as 1 to 4 scales indicating positive cytoplasmic and nucleus reaction in 1-25, 26-50, 51-75, and 76-100% of the cells. Evaluation of IHC staining slides was based on signal intensity and carried out using scoring system (*10-12*) .

The total scores for every spot in slides were recorded as the sum of scores for percentage of positive cells and staining intensity. The intensity of reaction in the procedure varied from gene to gene. All slides were examined at least three times for presence of the genes.

2.3. *Statistical analysis*

The data of IHC were stored and analyzed by SPSS version 12 software (SPSS Inc, Chicago, IL, USA). The protein expression of p16 and cyclin D1 genes data were evaluated non-parametrically using Mann-Whitney test. Tests were considered significant when p values were less than 0.05 ($p < 0.05$).

3. Results

Samples were obtained from twenty-one SCC patients. In analysis, tissues were compared with control tissues to enable complete analysis. The all SCC tissue samples revealed increasing protein expressions of p16 and cyclin D1 genes. The immunohistochemical study showed that protein expression of cyclin D1 increased in the nuclei of tumoral cells as compared with normal skin tissues. The p16 protein was expressed in the nuclear and cytoplamic areas of tumor cells.

In IHC method using the SCC tissues of human skin, cyclin D1 and p16 were significantly increased ($p = 0.004$ and $p = 0.019$, respectively). Correlation between protein expression of cyclin D1 and p16 genes in these samples was not shown, which suggested that these genes might work independently ($p = 0.931$). Depending on the genes, the staining was observed in the nucleus, cytoplasm or both. Staining intensity of cyclin D1 varied from +1 to +3, while that of p16 was from +2 to +3. Protein expression of cyclin D1 and p16 in SCC tissues were higher than those in normal

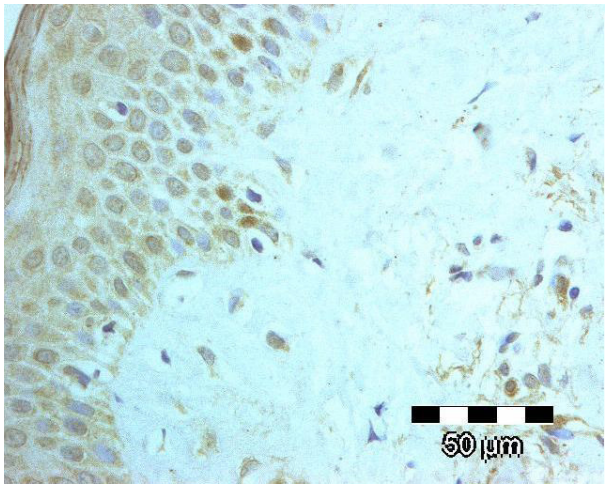

Figure 1. Immunostaining analysis showing nucleus and cytoplasm expression of p16 in TMA sample of normal skin tissue. The nuclei and cytoplasm of epidermal cells show brown staining at moderate intensity (+2). Magnification, 40×.

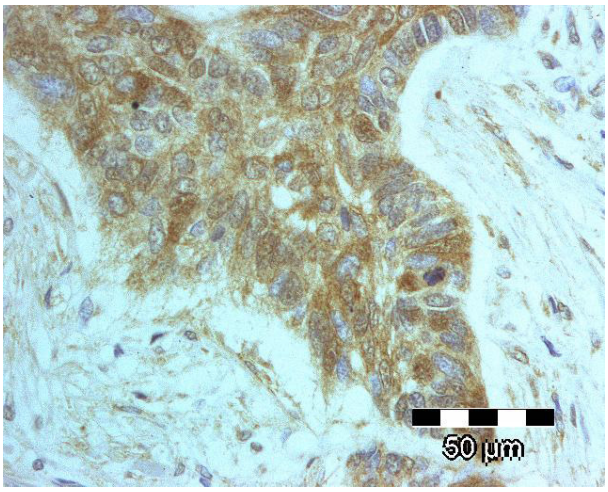

Figure 2. Immunohistochemical study for p16 on SCC of TMA sample. Strong brown staining intensity (+3) is shown in the nuclei and cytoplasm of the cells in SCC of skin. Magnification, 40×.

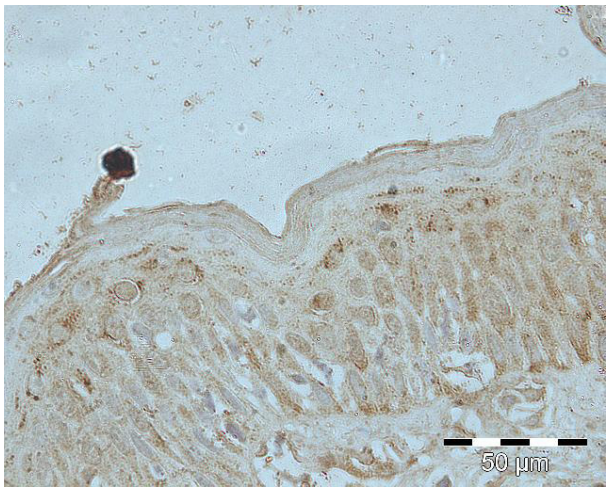

Figure 3. Immunostaining analysis of $CCND_1$ on normal skin tissue. Mild brown staining (+1) is illustrated in nuclei of normal epidermal cells. Magnification, 40×.

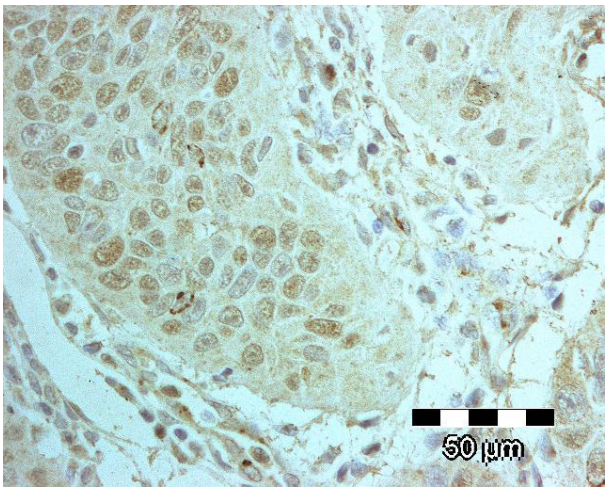

Figure 4. Immunohistochemical study on TMA sample of SCC tested for $CCND_1$. Moderate brown staining is shown in nuclei of the cells (+2). Magnification, 40×.

skin tissues.

IHC analysis demonstrated the protein expressions of p16 and cyclin D1 in both SCC and normal skin tissues. Figures 1 and 3 showed the protein expressions of p16 and cyclin D1 genes, respectively, in the normal skin tissues. Figures 2 and 4 showed the protein expressions of p16 and cyclin D1 genes, respectively, in the SCC tissues. Compared with the normal skin tissue, the protein expressions of p16 and cyclin D1 in the cells of malignant tissues were significantly higher.

4. Discussion

TMAs are used like whole tissue sections for immunohistochemical studies of the human SCC tissues. Two SCC cores are representative of whole tissue of one case in assessing expression patterns of proteins for cyclin D1 and p16 in this study. P16 and cyclin D1 proteins were expressed in all SCC samples which demonstrated the high sensitivity and specificity of this approach.

The present study assessed protein expression of these genes in SCCs and explained their different expressions in the malignant and normal human skin tissues. P16 and cyclin D1 genes are important in controlling cell proliferations in the normal and malignant tissues. The current study suggested that the changes of cyclin D1 gene altered the cell cycle proliferation. The high level of cyclin D1 protein in SCCs might result in the development of cancerous in skin tissue (*13,14*).

Over-expression of cyclin D1 in tumoral skin lesions in the other studies revealed the relevance and pathogenesis of this gene in skin carcinogenesis. Recent studies have provided evidence suggesting that

disruption of cyclin function might play a critical role in the tumorigenesis (*7,14-19*).

Meanwhile, the present study demonstrated the expression of p16 gene in the SCCs and normal skin tissues. P16 is an important gene in the cells of normal and tumoral tissues in the proliferation process. P16 protein expression in the SCCs was higher level of staining intensity presentation rather than the normal skin tissues (*17,18,20*) unlike the expected low intensity of staining which has been noted in the other cancers (*21-23*).

P16 gene might function as general regulator which mediates the switch between high proliferation and low proliferation in the cells (*23*). The acquired results from the current study like the other studies supported the role of p16 gene as a factor causing tumors (*2,23-25*).

The results showed the protein expression of these genes was detectable in all SCC tissue samples but the intensity is different from tissue to tissue. The detection of p16 and cyclin D1 genes has thus considerable potential as an assay to detect occult metastatic tumor cells from SCC and other neoplasms. p16 and cyclin D1 are not markers of SCCs but they can predict the outcome of management of the disease. Gene therapy can potentially control cell proliferation which is the essential process in cancer development.

Our results and those from other authors support the argument of demonstrating enhanced signals for multiple genes in malignant tissue and identifying the presence of small numbers of infiltrative deposits of SCC cells in the tissues. Taken together, the p16INK4 plays an important role in the skin carcinogenesis. The highly up-regulation of p16INK4 mediates growth control which likely contributes to the benign behavior of the tumor type. In neoplasm, the high level of p16 gene seems to activate the spontaneous regression process though the exact mechanism of this regulatory function needs to be determined. On the other hand, the loss of p16 gene represents a crucial step in the progression of SCC (*7*).

5. Conclusion

Our findings demonstrated the over-expression of cyclin D1 and p16 proteins in SCC tissues using IHC method. The over-expression of these genes probably induces the cell cycle proliferation which may result in the SCC in skin tissues. In conclusion, the present study provided a clear evident that the protein expression of cyclin D1 and p16 genes was highly detected in the SCCs as compared with normal skin tissues. Our results indicated functional genes in Rb pathway such as p16 and cyclin D1 which probably mediates the phenotype changes from normal proliferation to abnormal proliferation activity in the SCC cells.

The data implicated that cyclin D1 and p16 genes might be candidates for further evaluation as therapeutic targets in this cancer. This project showed that IHC is a sensitive method to assess protein expression of the genes. IHC presents the structure of tissue well to detect the small number of cancerous cells. The current study demonstrated the value of IHC using TMA as pre-screening tool in selecting patients suitable for analysing the alteration of cyclin D1 and p16 genes in diagnostic and research settings, respectively.

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Original Article

Exhaled ethane concentration in patients with cancer of the upper gastrointestinal tract – a proof of concept study

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Summary **There has been growing interest in the measurement of breath ethane as an optimal non-invasive marker of oxidative stress. High concentrations of various breath alkanes including ethane have been reported in a number of malignancies. Our aim was to investigate the use of novel laser spectroscopy for rapid reporting of exhaled ethane and to determine whether breath ethane concentration is related to a diagnosis of upper gastrointestinal malignancy. Two groups of patients were recruited. Group A (n = 20) had a histo-pathological diagnosis of either esophageal or gastric malignancy. Group B (n = 10) was made up of healthy controls. Breath samples were collected from these subjects and the ethane concentration in these samples was subsequently measured to an accuracy of 0.2 parts per billion, ppb. Group A patients had a corrected exhaled breath ethane concentration of 2.3 ± 0.8 (mean ± SEM) ppb. Group B patients registered a mean of 3.1 ± 0.5 ppb. There was no statistically significant difference between the two groups (p = 0.39). In conclusion, concentrations of ethane in collected breath samples were not significantly elevated in upper gastrointestinal malignancy. The laser spectroscopy system provided a reliable and rapid turnaround for breath sample analysis.**

Keywords: Ethane gas, oxidative stress, laser spectroscopy, esophageal cancer, gastric cancer

1. Introduction

Oxidative stress is a state in which the rate of formation of highly reactive free radicals exceeds the rate of removal resulting in a wide range of complex redox reactions that mediate biomolecular injury (*1*). Free radicals cause degradation of polyunsaturated fatty acids (PUFAs) by a well-researched process known as lipid peroxidation (*2*). The by-products of this intricate chain reaction include aldehydes (*e.g.* malondialdehyde), conjugated dienes (*e.g.* isoprene) and alkanes such as ethane and pentane (*3-6*). These products have been used to gauge the progress of lipid peroxidation *in vitro* and *in vivo*.

There is evidence showing high activity of several anti-oxidant enzymes and deficiency of anti-oxidant vitamins in a variety of cancers (*7-11*). In addition, several studies have explored the significance of lipid peroxidation products in a number of malignancies. High malondialdehyde levels have been found in esophageal, gastric and colorectal tumor tissue (*7*), as well as in the blood of patients suffering from breast (*9*) and gastric (*10,12*) cancer. On the other hand, breath testing studies have reported abnormally high concentrations of volatile organic compounds (VOC's) in lung (*12-14*) and breast cancer patients (*15,16*).

Backed by this evidence we set out to determine whether the exhalation of ethane (as a marker of oxidative stress *via* lipid peroxidation) is associated with upper gastrointestinal malignancy. Esophageal

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and gastric cancers have a notoriously bad prognosis when discovered late. At present, a curative strategy depends on diagnosis of early lesions that are amenable to radical surgical treatment. Therefore, this provides the rationale for research into the field of diagnostics in order to identify screening tests that can reliably detect early cancer.

2. Materials and Methods

2.1. *Subjects*

The Research and Ethics Committee of the North Glasgow University Hospitals NHS Trust gave ethical approval for this study. Twenty subjects with endoscopic and histo-pathologic evidence of upper gastrointestinal malignancy were recruited and constituted Group A in this study. Seven of these patients had adenocarcinoma of the esophagus and another 6 had squamous carcinoma of the esophagus. These patients had AJCC Stage II to III tumors. The remaining 7 patients had UICC Stage III to IV gastric adenocarcinomas. A further 10 subjects of similar age were recruited to serve as healthy controls, making up Group B. These subjects were selected from a University of Glasgow database for ethane levels in healthy adults. These controls had no history of gastrointestinal tract disease.

For the purpose of our study, the following were used as exclusion criteria in subject selection: i) anti-oxidant (including vitamin C, E and selenium supplements) medication; ii) steroid medication; iii) severe cardio-pulmonary disease precluding the collection of an adequate breath sample.

2.2. *Samples*

Three-liter Tedlar® bags with on-off valves were used for breath sample collection. These bags were flushed with hydrocarbon-free air (HCFA) (ethane < 100 parts-per-trillion) before being evacuated for breath sampling. In Group A, the samples were collected prior to surgical resection or endoscopic ablation of the tumors. In both groups subjects were asked to exhale into these bags in order to fill them up after having spent at least 30 min in their respective ward or clinic. We considered this to be the minimum time interval required for subjects to equilibrate with the ambient air (derived from our own unpublished data on healthy volunteers). Since cigarette smoke has been shown to significantly affect breath alkane measurements, samples from smokers were obtained at least 30 min after smoking to allow attainment of equilibrium, although it is accepted that this timescale may be too short in order to completely eliminate noise due to the smoke effect. In addition, one-liter Tedlar® bags were used to sample the ambient air for each subject recruited. Samples were processed typically one day after collection in order to minimize errors caused by gas diffusion through the sample bags (*1*).

2.3. *Measurement of ethane concentration*

The average ethane concentration in the breath samples [e_{sam}] and the average ethane concentration b in the ambient air were measured using an ultra-sensitive tunable diode laser spectrometer (TDLS). This device (developed at the Department of Physics and Astronomy, University of Glasgow) comprises a cryogenically cooled lead-salt laser diode probing a target gas sample flowing through a multi-pass optical cell. The instrument is highly specific to ethane gas and reports ethane concentration with accuracy typically < 0.2 ppb and essentially in real time. The molecular vibrations excited in ethane by the laser are known absolutely and subsequent calibration is very reliable. The system is controlled by an integrated laptop computer and custom-designed software. Each sample could be measured in around 8 sec and an example trace for a breath sample opened up to the instrument is shown in Figure 1. In contrast, gas chromatographs or gas chromatography and mass spectrometry techniques require longer measurement periods and sample concentration for the ethane levels involved here. Ethane concentration is expressed here in parts per billion (ppb). The corrected average exhaled ethane concentration $[e]_{cor}$ in the exhaled breath of our study subjects was calculated using the simple formula: $[e_{cor}] = [e_{sam}] - [b]$.

2.4. *Statistics*

An SPSS version 9.0 statistical software package was

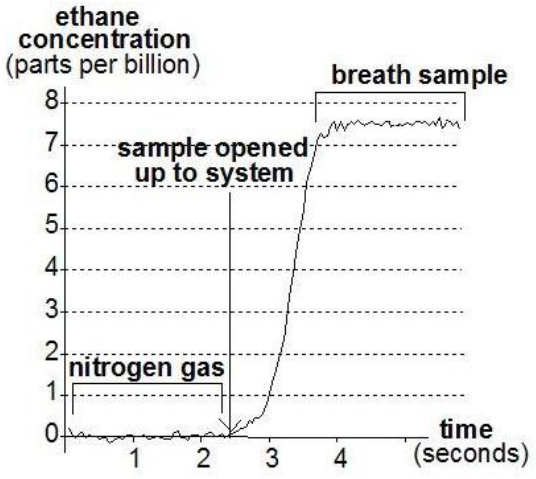

Figure 1. A typical trace of ethane gas in a breath sample from a patient in the study as measured by the TDLS. Note that this is the raw sample measured by the instrument and ambient background is not yet subtracted. The graph shows how rapidly the system registers the ethane in the sample bag. The deviation in the measurement at the plateau constitutes the intrinsic accuracy of the technique (around 0.2 ppb).

used to analyze our results. *P* values less than 0.05 were considered statistically significant.

3. Results

The observations and results for the two groups are summarized in Table 1. When compared for age, gender, a history of COPD and smoker status, there was no significant difference between the two groups (using Fisher's exact and Mann-Whitney tests as appropriate). As regards the corrected exhaled ethane concentration, there was no statistically significant difference between the two groups (Mann-Whitney test, $p = 0.39$). Likewise when the study population was divided into 4 subgroups according to cancer type (3 subgroups) and control status, there was no significant difference in ethane concentration (Kruskal-Wallis test, $p = 0.48$).

Figure 2 is a plot of $[e_{cor}]$ and age in the entire study population. The plot shows that there is no appreciable difference between patients and controls. It appears that with increasing age there is a downward trend in exhaled ethane concentration, however this observation did not reach statistical significance (Spearman's rank test, $p = 0.31$). We found no significant difference in ethane concentration between subjects with or without a history of COPD. Likewise there was no difference between smokers and non-smokers. The only significant difference was recorded when time from the last cigarette smoked was taken into account. Patients who had smoked 30-60 min before breath sampling were found to have a higher ethane concentration than non-smokers and/or those smokers who had abstained for more than 1 h before sampling (Kruskal-Wallis test, $p = 0.03$). Exclusion of the former group of smokers from our analysis of the difference in $[e_{cor}]$ between Groups A and B, was still not conducive to statistical significance (Mann-Whitney test, $p = 0.26$).

4. Discussion

Ethane (C_2H_6) is considered to be one of the best markers of lipid peroxidation (*2*). The main endogenous source of ethane in the body is peroxidation of n-3 PUFAs, specifically the cleavage of the 16-hydroperoxide of 9,12,15-linoleic acid. Conversely, pentane (C_5H_{12}) is evolved from degradation of the more abundant n-6 PUFA's. Both gases are saturated, aliphatic hydrocarbons, which are sparingly water-soluble. Ethane has a lower molecular weight that renders it poorly lipophilic as well. This also renders ethane more inert than pentane; it is less tissue-soluble and less likely to be metabolized. Ethane levels, therefore, exhibit less day-to-day variation than pentane. Preliminary studies have shown typical day to day variation of exhaled ethane in healthy adults in this age group to be in the range of 0.5 ppb compared to a mean of around 3 ppb (*19*). For this reason ethane was our preferred marker for oxidative stress.

Ethane is present in the air at around 1.5 ppb concentration in the Northern Hemisphere. For any exhaled breath sample, a fraction of the ethane level will be due to this environmental component. Since our subjects were submitting expiratory volumes from total lung capacity (TLC) to residual volume (RV), the dead space volume introduced an exogenous ambient ethane component causing a systematic error. Contrary to other studies employing HCFA breathing techniques or dead-space eliminating sampling methods, we opted for an ambient air breathing method and a simple bag sampling technique (*20*). In order to correct the error introduced by the inhaled ethane fraction, we firstly allowed the subject to come into equilibrium with the ambient air before breath sampling. Subsequently, we subtracted the ambient ethane concentration from the exhaled breath sample concentration. We consider our strategy to be simple, straightforward and adequate for the purpose of a proof of concept investigation. In this study we accepted breath samples only where the accompanying ambient level was less than 5 ppb in order to keep a significant proportion of breath ethane over ambient level and reduce errors.

Table 1. Summary of the results

	Group A (cancer)	Group B (control)
Number of subjects	20	10
Mean age, yrs ($p = 0.29$)	72 ± 2	67 ± 4
Male : Female ratio	2.3:1	2.0:1
Smokers ($p = 0.68$)	30%	20%
COPD ($p = 0.63$)	30%	10%
Mean of corrected average exhaled ethane concentration $[e_{cor}]$, ppb ($p = 0.39$)	2.3 ± 0.8	3.1 ± 0.5

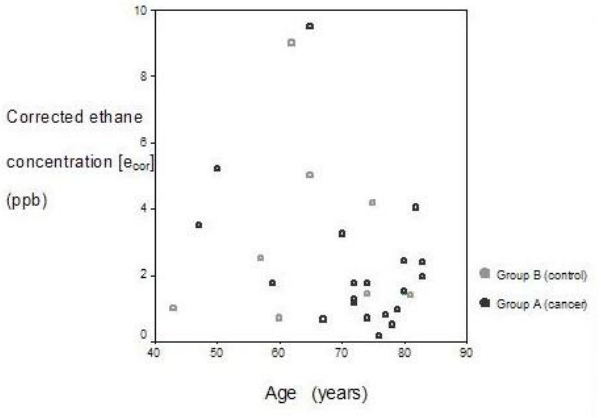

Figure 2. Ethane concentration $[e_{cor}]$ and age. There is no appreciable difference in $[e_{cor}]$ between patients and controls. There appears to be a downward trend with age but this was not statistically significant ($p = 0.31$).

Abnormally high breath ethane levels have been reported in several disorders including smoking (*21*,*22*), asthma (*23*), chronic obstructive pulmonary disease (COPD) (*24*,*25*), cystic fibrosis (*26*), myocardial infarction (*2*), inflammatory bowel disease (*27*,*28*), HIV infection (*29*). Interestingly, elevated concentrations have also been noted in less well-understood conditions such as multiple sclerosis (*30*), schizophrenia (*31*), and childhood attention deficit (*32*). To date ethane has not been employed in cancer studies. High concentrations of o-toluidine were detected in association with several tumours (*33*). Pentane was found to be abnormally high in a breast cancer (*15*). Another breast cancer study used a set of VOC's and reported that a three-dimensional display of the alveolar gradients of these gases had a better negative predictive value than actual mammography (*16*). In one lung cancer study, butane was identified as the best single discriminator for lung cancer (*12*).

Our results failed to show a useful association between exhaled ethane concentration and invasive upper gastrointestinal cancer. This result is significant because there is ample evidence that these conditions are indeed linked with the state of oxidative stress. The only significant elevations we recorded occurred soon after smoking and this observation has already been reported (*21*). This phenomenon is probably due to damage of the respiratory mucosa mediated by free radicals in cigarette smoke (*22*). We also note that the mean ethane concentration in our control group (3.1 ± 0.5 ppb) was higher than was recently reported by Paredi and colleagues (0.88 ± 0.09 ppb) (*25*,*26*). However, the control population in these studies was significantly younger than our own (mean age 33 and 74 years, respectively).

5. Conclusion

This is the first study employing a tunable laser-diode spectrometer to explore the relationship between upper gastrointestinal malignancy and ethane concentration in exhaled breath. We conclude that analysis of breath ethane based on a simple bag collection procedure and after taking account of ambient conditions, does not provide a useful discriminator for invasive esophageal and gastric cancer.

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Original Article

Effects of premedication of midazolam or clonidine on perioperative anxiety and pain in children

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Summary **The aim of the present study was to compare clinical effects of oral midazolam and clonidine premedication in children. In a prospective randomized double blind trial, 45 children between 2-8 years old received either oral midazolam 0.5 mg/kg (group M) or clonidine at 2 μg/kg (group C_2) or 4 μg/kg (group C_4). The level of sedation, quality of parental separation, mask acceptance and thermodynamics were recorded. Postoperative analgesia, and perioperative side effects were assessed. In comparison to group M, the scores of sedation, parental separation and mask acceptance were significantly higher in group C_2 and group C_4 ($p < 0.05$). Also the level of sedation was significantly better in group C_4 than in group C_2 ($p < 0.05$). However, the rate of postoperative analgesia decreased significantly in group C_2 and C_4 compared to group M. The incidence of shivering was significantly increased in group M compared to group C_2 and C_4. Oral clonidine premedication can therefore provide better preoperative sedation and postoperative analgesia with few adverse effects.**

Keywords: Clonidine, midazolam, children, premedication

1. Introduction

Preoperative anxiety and pain are known to lead to maladaptive behavior in the postoperative period (*1*). A multimodal approach consisting of sedative drugs, parental presence, play therapy, familiar environment and effective pain therapy is necessary to reduce preoperative anxiety (*2,3*).

Oral premedication is widely used in pediatric anesthesia to reduce preoperative anxiety and ensure smooth induction. The benzodiazepine midazolam, an anxiolytic drug, is the most commonly used premedication (*4,5*). Midazolam has a number of beneficial effects when used as premedication in children: sedation, reduction of vomiting, fast onset and limited duration of action (*6,7*). However, it is far from an ideal premedication, having an increased incidence of adverse effects such as postoperative behavior changes, cognitive impairment (*8*), paradoxical reactions, and repiratory depression (*9*). Recently, new drugs such as the alpha 2-agonists have emerged as alternatives for premedication in pediatric anesthesia. Clonidine, a selective centrally acting partial alpha 2-agonist, has been reported to improve perioperative hemodynamic stability, provide analgesic properties, and decrease anaesthetic requirements (*10-12*). However, the reports about administrated dosage of clonidine have remained inconsistent (*13,14*).

The main aim of this study was to evaluate and compare the influence of different premedication regimens on preoperative sedation, separation from parents, and mask acceptance in children. We also compared postoperative analgesia, hemodynamic status, and adverse effects.

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2. Materials and Methods

2.1. *Case selection and study design*

After approval of the Hospital's Ethics Committee

and parental written informed consent, 45 children, ASA status I or II, aged between 2 and 8 years, and undergoing selective ventriculoperitoneal shunt were included in this double-blind, prospective, randomized study. All children exclude abnormal liver function and renal and mental disease.

Children were randomized into the following three groups: group M (n = 15), group C_2 (n = 15), and group C_4 (n = 15) received midazolam 0.5 mg/kg, clonidine 2 μg/kg, and clonidine 4 μg/kg given orally 60 min prior to anesthesia induction, respectively. The premedication was mixed with 5 mL syrup. All children who refused to take the premedication or spat it out were excluded from the study protocol.

2.2. *Assessment and data collection*

Heart rate (HR), blood pressure (BP), pulse oxygen saturation (SPO2), and respiration rate (RR) of children were monitored routinely during perioperation. Ringer's lactate solution was infused according to the child's weight. Preoperative level of sedation was assessed by using a 3-point scale: 1 = crying and struggle, 2 = alert, 3 = drowsy. Parental separation was evaluated by using a 3-point scale: 1 = anxiety and struggle, 2 = anxiety, easily calmed, 3 = drowsy and calm. A 4-point scale was applied for evaluation of mask acceptance (*3*): 1 = combative, angry, 2 = fear of the mask, not easily calmed, 3 = fear of the mask, easily calmed, 4 = calm, cooperative. Assessment was performed by a consultant anesthetist who had no knowledge of the type of premedication.

A strict anesthetic protocol was applied. Anesthesia was induced with inspired isoflurane 2.5% in a fresh gas flow of 3-litre/min. The maintenance of anesthesia was carried out with inspired isoflurane 1.1%~1.5% combined with remifentanyl (0.2 $\mu g \cdot kg^{-1} \cdot min^{-1}$) and vecuronium (0.08 $mg \cdot kg^{-1} \cdot h^{-1}$) given intravenously. If children complained of pain, frequent crying, and dysphoria after operation, remedy analgesia was provided by a rectal loading dose of paracetamol of 30-40 mg/kg. All adverse effects including hypotension (SBP < 70 mmHg), bradycardia (HR < 60 beats/min), respiratory depression, nausea/vomiting, and shivering were recorded and administered in the perioperative periods.

2.3. *Statistical analysis*

Results were analyzed using SPSS10.0 software, and all values were reported as mean plus/minus SE. Data analysis for measurement data was performed by unpaired Student's *t*-test to detect the differences between the groups for age, weight, operation time, duration of anesthesia, and various scores. Numerical data was analyzed using the chi-square test. A $p < 0.05$ was considered statistically significant.

3. Results

The three groups were similar with respect to age, weight, gender, operation time and duration of anesthesia (Table 1).

Table 2 summarizes preoperative scores and postoperative analgesia for the three groups. In comparison to group M, the scores of sedation, parental separation and mask acceptance were significantly higher in group C_2 and group C_4 ($p < 0.05$). It is to noted that the level of sedation was significantly better in group C_4 than in group C_2 ($p < 0.05$). However, the rate of remedy analgesia decreased significantly in group C_2 and group C_4 than in group M.

Table 3 shows adverse effects of various groups in the perioperative period. The incidence of shivering was significantly increased in group M than in group C_2 and C_4 ($p < 0.05$). No significant differences were observed between clonidine and midazolam in hypotension, bradycardia, respiratory depression, and nausea/vomiting.

4. Discussion

This study demonstrates clinical advantages of oral clonidine, in both the preoperative sedation and postoperative analgesia, compared with oral midazolam when used as a premedication in children.

Clonidine is experiencing increasing use in the pediatric population as a sedative and analgesic because

Table 1. Patient data

	Group M	Group C_2	Group C_4
Age (year)	5.0 ± 2.1	4.8 ± 1.9	5.2 ± 2.3
Gender (male/femal)	8/7	8/7	9/6
Weight (kg)	17 ± 5	16 ± 6	17 ± 6
Operation time (min)	65 ± 21	62 ± 27	65 ± 22
Anesthesia time (min)	68 ± 28	65 ± 30	68 ± 31

Data are expressed as mean ± SE, $p > 0.05$.

Table 2. Sedation, parental separation and mask acceptance scores and remedy analgesia: midazolam compared with clonidine

Groups	Sedation score	Parental separation score	Mask acceptance score	Remedy analgesia (%)
M	2.1 ± 0.5	1.6 ± 0.5	1.4 ± 0.6	80
C_2	2.4 ± 0.6*	2.1 ± 0.6*	2.8 ± 0.8*	20*
C_4	2.7 ± 0.4*#	2.2 ± 0.6*	2.9 ± 1.0*	20*

Data are presented as mean ± SE; * $p < 0.05$ in comparison with group M; # $p < 0.05$ in comparison with group C_2.

Table 3. Adverse effects of various groups in perioperative period

Groups	Hypotension	Bradycardia	Respiratory depression	Nausea/ vomiting	Shivering
M	0	0	0	3	6
C_2	0	1	0	1	1*
C_4	2	1	0	1	0*

Data are presented as mean ± SE; * $p < 0.05$ in comparison with group M.

of its central alpha2-adrenergic agonist capability (*15-17*). Results of this study indicate that sedation and the anti-anxiety effect of clonidine might contribute to activation of alpha-2 adrenergic receptors in locus coeruleus or in the frontal lobe cortex (*18*).

One major drawback of clonidine used as a sedative premedication is its slow onset of action (*19,20*). So, oral clonidine needs to be administered at least 60 min prior to induction whereas satisfactory sedation can be achieved 30 min after oral midazolam. However, anesthetists may prefer to accept the delay in onset of sedation of clonidine if clinical advantages occur during the perioperative period.

The effect of sedation was found to be related to dosage of clonidine premedication in children for ophthalmic surgery (*21*). Schmidt *et al*. (*22*) reported that children receiving clonidine premedication have similar levels of anti-anxiety and sedation as those receiving midazolam. In contrast, our study demonstrated clonidine premedication provided better levels of sedation and anti-anxiety in children than those given midazolam.

A prospective study of patients undergoing outpatient laparoscopic cholecystectomy indicated patients receiving clonidine (0.15 mg orally 60 min before surgery) required less additional analgesia (*23*). In this study, children given clonidine had less postoperative pain than those given midazolam. However, it remains unclear if this effect is related to activation of postsynaptic α2 receptors in the descending noradrenergic pathway of the spinal cord or related to an anxiolytic effect not measured (*24*).

Mikawa *et al*. showed oral 5 μg/kg clondine premedication led to severe bradycardia, and needed atropine treatment (*25*). The study selected clonidine at a dosage of 2 μg/kg and 4 μg/kg concomitant with atropine intramuscular injection. Only one child in each clonidine group had bradycardia. Likewise, there were no differences in the incidence of hypotension, nausea/vomiting, respiratory depressing between clonidine and midazolam groups. A previous study suggested clonidine was effective treatment for postananesthetic shivering (*26*). In the present study, the incidence of shivering was lower in the clonidine group than in the midazolam group. The mechanism of clonidine preventing shivering was correlated with inhibition of vasoconstriction and a decrease of shivering threshold (*27*).

In conclusion, in this study, premedication with oral clonidine appeared to be superior to oral midazolam. Oral clonidine premedication provided better sedation, anti-anxiety, postoperative analgesia, and prevented postoperative shivering with few adverse effects.

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BioScience Trends

Guide for Authors

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