Our experience of ancient DNA study in Korea

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Ancient DNA

DNA study of ancient human samples (aDNA study) is regarded as a newly emerging research field, attracting keen attention from the scientific community (Marota & Rollo 2002). aDNA study can provide crucial genetic information on bodies buried in ancient tombs or even on genetic lineages of human populations, information of which could not easily be derived by other studies (Iwamura et al. 2004).

Since the first report on ancient DNA (aDNA) studies using ancient samples from animal and human remains, molecular biological technique has become common applied to archaeological science (Willerslev and Cooper, 2005). Considering that there are very few copies of aDNA remained in ancient tissues, polymerase chain reaction (PCR) became very helpful technique to bioarchaeologists. With the invention of the PCR technique, the sensitivity of aDNA study was dramatically improved; therefore, there were many early reports on ancient DNA extracted from several thousand or even million year old samples. However, regretfully enough, it became evident that many of such reports were in fact the outcome of contaminating modern DNA (Melchior et al., 2008).

Actually, most ancient specimens were known to possess, if any, only contain amplifiable endogenous DNA fragments sized 100–500 bp (Willerslev and Cooper, 2005). Since endogenous DNA remained in ancient samples was thought to be easily decayed during long enough time of burial, bioarchaeologists generally failed to get any meaningful aDNA data from them (Hofreiter et al., 2001).

Therefore, researchers should have cared about the increased contamination risk of modern DNA as well because PCR also made unhopeful amplification of DNA from environment. Anyway, human DNA is prevalent anywhere in archaeological sites, storing places and wet lab etc. Considering that modern human DNA sequences can readily be retrieved from ancient remains, some researchers even believe that many previously reported studies on human aDNA sequences are unreliable (Hofreiter et al., 2001). Actually, some of great achievements in early history of aDNA analyses were thought to be the outcome of obvious human or microbial contamination (Willerslev and Cooper, 2005).

Therefore, if we did not take an effective step for making our aDNA study much authentic, the sequence data could not be regarded as endogenous DNA from the ancient samples. To minimize such a possible contamination of modern DNA, researchers developed a number of criteria that should have been respected for making their authentic aDNA analyses much authentic. The criteria aim at making the samples not to be contaminated until lab work starts; and the aDNA lab itself not to be so seriously contaminated by any modern DNA. Even if DNA contamination would be inevitable during experiment, the origin of aDNA should be clearly defined. Briefly, we should know the DNA profile of every aDNA researchers joining in the work. Comparing their DNA profiles with those obtained from ancient samples, we

could rule out the possible contamination of modern DNA in them. The other detailed provisions are summarized in various criteria for authentic aDNA analyses (Willerslev and Cooper, 2005; Hofreiter et al., 2001).

Many provisions were developed for successful aDNA study. Protection clothing such as gowns, head caps, gloves, and masks was sterilized to be worn by researchers while they did aDNA analyses on the samples. Though this could be useful for minimizing modern DNA contamination during lab work, it was not sufficient because the crucial problem concerning aDNA analysis is the abundance of modern human DNA in the lab. Anyway, some researchers even thought that archaeological manipulation of human remains could be another source of modern DNA contamination (Melchior et al., 2008). To get the samples in which modern DNA contamination could be minimized, they removed the last layer of soil from the skulls by themselves, supporting the absence of prelaboratory modern DNA contamination.

Briefly, researchers commonly recommended that the preparation of aDNA amplification should be done in a laboratory that is only dedicated to aDNA analysis. PCR with modern DNA has been strictly restricted in the work place where aDNA analysis was performed. In general, the lab for aDNA extraction and preparation for PCR was done in the building separated from workplace where PCR amplification was performed. Yoder and Delefosse (2002) also suggested the ideal clean room design. Briefly, the independent lab should be dedicated to aDNA work, which must be separated from the main lab. According to them, aDNA should be composed of three independent chambers: an ante-room for changing the clothing for clean room work; a small PCR preparation room, and an aDNA extraction room. They recommended that all three chambers must be equipped with positive pressure filter system. The chambers should be routinely exposed to short-wave UV light (Yoder and Delefosse, 2002).

Our Experience for Ancient DNA Analysis

Researchers from European countries reported the osteometric data about Koreans after the country established diplomatic relations with European and American countries in the end of 19th century (Chang, 1979). The trend was changed when Korean Empire was annexed by Japanese in 1910. After then on, most of anthropological studies on Koreans were only performed by Japanese anatomists: among 370 or more anthropological studies on Koreans published until 1945, 72.5% of the articles were written by Japanese scholars while only 17.4% of papers were reported by Korean researchers (Chang, 1979).

Though anthropological works started to be performed by Korean anthropologists after World War II, lack of experiences was blocking progress in the research field of Korea. Like western countries (Mays, 2008), in 1950s–1980s, most of osteologists in Korea were trained under medical backgrounds. Even if physical anthropologists started to come from archaeological backgrounds in 1980s, most of the studies were concentrated on the bones of contemporary Koreans maintained in medical school, but not on the human skeletons discovered in archaeological fields of Korea.

However, in 1990s, various types of biological techniques have been newly added to the conventional studies on the samples from archaeological fields. In case of countries leading the archaeological sciences, covering the period 1991-2007, increase in bone chemistry have made some shifts of emphasis in the proportion of anthropological articles. The studies devoted to stable isotope and ancient DNA has become particularly common in those countries (Mays, 2008). The situation also started to be changed in Korea nowadays.

There were new trends in anthropological studies using biological samples from excavation fields of Korea. The researches in Korea have extended their works beyond the osteometric studies, even to bioarchaeological works such as stable isotope, or ancient DNA analysis etc. We also performed a number of studies on aDNA included in ancient samples from archaeological sites in Korea. First of all, we did extraction and sequencing of parasite aDNA by PCR amplification with ancient samples. aDNA sequence of *Trichuris trichiura* obtained from samples of Joseon tombs showed 100% homology to that of the small subunit ribosomal RNA (SSUrRNA) gene of modern *T. trichiura*. The aDNA of another nematode species, *Ascaris lumbricoides*, was also extracted and sequenced by us. We have reported the first *Ascaris* aDNA from a medieval Asian country; and thus will expand the scope of *Ascaris* aDNA research (Oh et al., 2010).

Our aDNA work was not restricted to DNA work of infectious diseases. In our study on multiplex autosomal short tandem repeat (STR) genotyping, we could obtain genetic information from ancient human samples. Briefly, we showed that the MiniFiler kit would be a useful complement to conventional STR kit analysis of ancient samples because large-sized STR markers from highly degraded aDNA were amplified very successfully by MiniFiler kit analysis (Oh et al., *in press*). We also showed that the amelogenin aDNA assay can be very significant to anatomists when employed in adjunct to conventional anatomically or culturally based sex determination. In the cases in which the cultural and anatomical findings were discordant in sex determination, the PCR-based amelogenin assay was used corroborate either the former or the latter (Kim et al., *in press*).

However, even if our previous reports promised the bright future of aDNA studies in Korea, as seen in the reports of other researchers, we should also care about the possibility that our aDNA analysis in some cases might not be the authentic DNA sequences of human or animals hundreds to even million years ago, but the outcome of modern DNA contamination.

To minimize such a possible contamination of modern DNA, we also adopted the strict measures that were enforced for our recovery of authentic DNA from ancient samples. We could not deny the importance of limiting the number of researchers involved, because this does seem to correlate with dramatic reductions in the possibility of sample contamination by modern DNA. Indeed, restricting access to essential personnel only, during the unwrapping or sampling procedure, can be decisive. Determining the reasonable number of participants, that which strikes the fine balance between protecting the authenticity of an aDNA study and having enough as well as the right kind of expertise on hand, seems to be very crucial to the success of aDNA researchers.

When the number of researchers joining in the experiment could be limited; and the genetic profiles of them were identified before aDNA analysis, it could be easily confirmed if the ancient samples were contaminated by modern DNA, especially by those from personnel participating in the research. Table 1 clearly showed how we could know whether our aDNA data were the outcome of possible contamination by modern DNA from researchers. In this study on ancient Korean skeleton from Bronze Age dolmen, PCR amplicons of ancient samples were thought to be originated from one of us, because some amplified sequences of aDNA were the same as those of researchers. If we did not follow the *rule for authentic aDNA analysis*; therefore, we could not find the possible contamination of the sample by modern DNA, those btained by PCR were regarded authentic aDNA sequences.

Of course, there are many other ways for removal of modern DNA contamination during aDNA analysis, especially in case that we discovered human remains superbly preserved in Joseon tombs. Our concept of sampling is very simple for these cases. If human remains had not been exposed to the outside when found, that is, if they were still encased in their own

microenvironment (e.g., the soils surrounding them or, as in the present case, clothing) (Bouchet et al. 2003), the collected human samples would render aDNA investigation and results much more authentic.

Clothing removal from Korean mummies in our lab is a good example. Sampling human remains from Joseon tombs, in fact, entails a number of merits from the perspective of authentic aDNA study, conditions that could not easily be met, if at all, in regard to the other types of tombs. First of all, since the coffin is completely sealed by the concrete-block-like shields, the mummified body is almost perfectly isolated from surrounding soils over the long duration of burial. Therefore, contamination or derangement would be minimized if the concrete-block-like shields could be maintained until we finally opened the Joseon tomb. This might be also meaningful if our subjects are parasite eggs or larvae, which could be frequently contaminated from the surrounding soils.

Next, isolation of a mummified body from the outside environment could be also fulfilled because the bodies were heavily clad in clothes when interred within the coffin. Further, many pieces of clothing were wrapped around or stacked upon the body. Therefore, if a Korean mummies discovered at an archaeological site would be moved directly to an aDNA lab without disturbance of the clothes, after which removal of the clothes were performed under contamination-minimized lab conditions, human samples could be obtained, for which the profiles of contacting people were perfectly identified by us; therefore, easily confirming modern-DNA-contamination of the samples.

Considering that we could do aDNA studies with the samples, for which particular worries about modern DNA contamination could be reduced, the value of our procedure in enabling the achievement of authentic aDNA work is very significant to us. A procedure similar to ours might also be applied to other archaeological cases, which is to say, those in which various types of microenvironments (i.e., in the present case, clothes wrapped around the body) have still protected human samples when they were discovered in archaeological sites. If such samples were moved to our lab while maintaining their microenvironment, and if they were uncovered under contamination-minimized conditions, authentication of aDNA studies could be enhanced.

Joseon Mummy Project: Future of aDNA work in Korea

Archaeological sciences of Korea, lagging behind the other countries, were also adversely affected by maintenance of ancient bone collections until quite recently. Owing to the poor preservation conditions (e.g. low pH etc.), Korean archaeologists were difficult to collect ancient human bones from archaeological sites. Except for the bones from tombs of Joseon Dynasty (1392-1910 AD) where human remains could be exceptionally well preserved, most bones from ancient or medieval Korean graves were found to be fragmentary; or in case that the complete set of the bones could be fortunately collected, DNA preservation status was quite poor, even comparing with those of neighboring countries such as Japan and China. Therefore, there were only 379 skeletal remains of ancient Korean people maintained in universities, institutes or museums. Considering that skeletal collections maintained in one country are vital for competent osteoarchaeological studies (Mays, 2008), failure in making bone collections during the past several decades was very serious to anthropologists in Korea.

To overcome this, during the past several years, we were in the process of establishing the collection of Joseon people skeletons, where human remains from Joseon tombs have been maintained in contamination-minimized status. Fortunately, even if the preservation condition of ancient samples in Korea was not so good in general, we started to know that the

mummified human samples still perfectly preserved at the time of discovery could be collected from some exceptional Joseon tombs, not so rare a phenomenon in the archaeological sites of Korea. Actually, as far as we know, the mummified human remains represent the best opportunities for anthropologists to study the health and disease statuses of Joseon Koreans.

Our work on Joseon mummies started as early as 2002, with '*Yangju*' child mummy, under the collaboration with Dankook University Museum. We observed Korean mummies' preservation status by morphological (Chang et al., 2008), radiological (Lim et al., 2008), endoscopic (Kim et al., 2006) and *post-factum* dissection (Lim et al., 2008) methods. Using magnetic resonance imaging (MRI) technique, high-quality-preservation status could be confirmed again for Korean mummy cases because MRI in fact was successfully applied even to differentiation of various organs and tissues from Korean mummy (Shin et al., 2010). Certainly, our previous studies have clearly shown that Korean mummies' various internal organs were preserved very well, a phenomenon only rarely observed in previous mummy studies.

Our previous Korean mummy studies were focused not only on scientific evidences for superb preservation status, but on the paleopathological subjects. We searched for any paleopathological evidence of disease in mummified or skeletonized human remains obtained in the archaeological field. The prevalence, for example, of diffuse idiopathic skeletal hyperostosis (DISH), affecting the spinal column and extraspinal ligaments and tendons, was observed less frequently in our Joseon samples than among other archaeologically-obtained specimens from European monastic sites (Kim et al., 2010).

We also reported a possible case of rheumatoid arthritis (RA) in an 18th century Joseon female sample, in which signs of late-stage RA were identified mainly in the peripheral skeleton (Kim et al., 2011). The prevalence and distribution of dental caries among the Joseon people was another subject we have explored. In 16^{th} - 18^{th} century teeth of Joseon people, the prevalence was lower than for other collections of similar chronology, which in part might be caused by the low refined-sugar consumption in Joseon society (Han et al., 2010). Evidence of traumatic injury among Joseon people was also reported by us. Upon examination of an elderly individual who had lived sometime during the 16^{th} - 17^{th} century, computerized tomography (CT) radiographs and *post-factum* dissection revealed signs indicative of a mandibular fracture (Lee et al., 2009).

Further to our own micro- and macroscopic morphological examinations, we note the recent emergence of DNA analysis as an effective paleopathological tool, which has attracted keen attention from researchers (Marota et al., 2002). The new research field is based on the fact that infectious or hereditary disorders leave genetic evidence in archaeologically obtained human remains, which important information is not easily derived by other means. Recently, Hawass et al. (2010) made very impressive contribution to paleopathological studies based on molecular-diagnostic approaches.

In 2007-2009, the studies under multidisciplinary collaboration between anthropological, radiological, and molecular biological fields were performed on Egyptian royal mummies (Hawass et al., 2010). Their study, as part of the *King Tutankhamun Family Project*, showed construction of the royal family pedigree by using Y-chromosomal genetic information. They also found that aDNA analysis could determine the familial relationships as well as the pathological features attributable to some inherited diseases among 11 royal Egyptian mummies (Hawaass et al., 2010). Although some controversial issues concerning the preservation of nuclear DNA in Egyptian mummies were touched off, this study was evidently attributed to the development of aDNA and paleopathology field.

Inspired by the achievement, we also plan to undertake a series of aDNA-analysis-based paleopathological studies on Korean mummies. Indeed, our plan is to conduct various biomedical studies on 10 Korean mummies, including aDNA studies, the results of which yielded crucial knowledge of possible infectious or inherited diseases afflicting the Joseon society. The researches, appropriately, will be called the *Joseon Mummy project* (JMP), which will be ongoing for the next several years. The Korean mummy cases we selected for examination were of a preservation status far superior to any other human remains discovered in Korea (Fig. 1). Preliminary morphological and radiological examinations had been already conducted to obtain basic biological information for them.

To see if Joseon mummies were suffered from any single-gene (Mendelian) disorders, which are inherited in a Mendelian pattern by way of a single gene mutation, we searched for the online version of Mendelian Inheritance in Man (OMIM), the database continually maintained by the National Library of Medicine (http://www.ncbi.nlm.nih.gov/omim). Based on the OMIM searching, specific genes implicated for Mendelian inheritance diseases will be analyzed, to which those for Diabetes Mellitus, Hyperlipidemia, Metabolic syndrome etc. belonged (Table 2).

Our JMP study will also focus on the infectious diseases that possibly affected the Joseon people. Drancourt and Raoult (2005) already summarized the candidate infectious diseases based on a previously reported data on infectious diseases identified by molecular diagnostics, in which different infectious pathogens (e.g. bacteria, virus, and parasite) were included (Table 3). Of these, *Mycobacterium tuberculosis*, *Treponema pallidum* and *Yersinia pestis* are also significant to us, given both that they are among the most frequently studied by paleopathologists. Since there has been very little information on the infectious diseases among the Joseon people, we will make a special effort to obtain any relevant aDNA evidence for them, in our JMP tissue samples.

Our JMP has an advantage over typical aDNA-based paleopathological studies. In general, aDNA researchers thought that PCR-amplified fragments could not be obtained after about 600 years of burial. Considering that our tissue samples are just 300-500 years old, our own PCR results are thought to be very reliable, reproducible, and suitable, therefore, as a basis for further investigations into genetic diseases of pre-modern Korean peoples. Considering that we could do aDNA studies with the samples, for which particular worries about modern DNA contamination could be reduced, the value of our procedure in enabling the achievement of authentic aDNA work is very significant to us.

In the present report, we summarize our findings and look forward to future paleopathological work on Joseon mummies. In any event, despite the many remaining, unresolved mysteries and difficulties relating to studies on Joseon diseases, we can be optimistic about the prospects for JMP, not least because the data thereby acquired, on which historians, archaeologists and medical scientists depend for their understanding of that society, are otherwise utterly unobtainable. Any queries on the contents of this article could be directed to cuteminjae@gmail.com (DH Shin). This study was supported by the National R&D project of the National Research Institute of Cultural Heritage, Cultural Heritage Administration (NRICH-1107-B09F-1).

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Figure 1
Some cases examined in Joseon Mummy Project.

Table 1
Haplotypes of sample and related researches
(positions 15992-16390; 35-371)

	Hypervariable region 1		Hypervariable region 2	
Samples	**15992-16219**	**16220-16390**	**35-219**	**220-371**
A1	CRS	ND	73G	ND
A2	16172C 16174T	ND	73G	ND
A3	16172C 16174T	16223T 16362C	73G	248d 263G 309.1C 315.1C
B1	16172C 16174T	CRS	73G	CRS
B2	16172C 16174T	16223T 16362C	73G	281G 309.1C 315.1C
B3	CRS	CRS	73G	CRS
RS1	16183C 16189C	16220C 16254G 16298C 16362C	73G	248d 263G 309.1C 315.1C
RS2	16172C 16174T	16223T 16362C	73G	263G 309.1C 315.1C
RS3	16182C 16183C 16189C 16217C	CRS	73G 146C 150T 195C	263G 315.1C
RS4	CRS	16223T 16234T 16362C	73G	263G

Samples, RS1-4; Researchers; A1-B3; d, deletion

Table 2

Possible Molecular Diagnostic Approaches for Inheritance Diseases in *Joseon Mummy Project*

Diseases	Target gene (area)	Related disease
Diabetes Mellitus	Insulin receptor substrate-1 (IRS-1), Mt16189	type 2 diabetes
Hyperlipidemia	low-density lipoprotein(LDL)	familial combined hyperlipidaemia
Leri-Weill dyschondrosteosis (short stature)	SHOX	short stature, a characteristic curving of the radius (Madelung deformity)
Metabolic syndrome (abdominal obesity)	CD36	Hyperlipidemia, type 2 diabetes, heart dieseas
Obesity	Leptin, PPAR-gamma, UCP-1	Type 2 diabetes mellitus, hypertension, cardiovascular disease, and some cancers
Osteoporosis	Estrogen receptor (ER) α & vitamin D receptor (VDR)	Vertebral fracture
Asthma	Interleukin 4, human signal transducer and activator of transcription 6 (STAT6)	Allergen-induced airway hyperreactivity
Atherosclerosis	low-density lipoprotein (LDL), apolipoprotein(apo)B, apoAII, apo(a), apoE-CI-CII, lipoprotein lipase	familial combined hyperlipidaemia
Hypertension	Angiotensinogen	Essential hypertension
Sudden cardiac death	KVLQT1, HERG, KCNE1, KCNE2, and SCN5A	Long QT syndrome(LQTS)
Colon cancer	Adenomatous polyposis coli (APC)	Familial adenomatous polyposis (FAP)
Gastric cancer	PSCA (prostate stem cell antigen)	Diffuse-type gastric cancer
Leukemia	Human BCR-ABL1	Lymphoblastic leukaemia
Lung cancer	p53, CYP1A1	Heavy smokers
Alzheimer disease	apolipoprotein E (APOE)	Dementia
Parkinson disease	α-synuclein, parkin, DJ-1, PINK1	Familial forms of Parkinson's disease
Personality disorder	Serotonin transporter (SERT), dopamine 4 receptor (DRD4)	Anxiety-related and novelty-seeking personality traits
Ankylosing Spondylitis	HLA-B27	Autoimmune disease
Rheumatoid Arthritis	Genes in the HLA complex	Autoimmune disease
Systemic Lupus Erythematosus (SLE)	TNIP1, PRDM1, JAZF1, UHRF1BP1, IL10	Autoimmune disease

Table 3

Possible Molecular Diagnostic Approaches for Infectious Diseases in *Joseon Mummy Project*

Diseases	Target gene (area)	Usual specimen
Bartonella quintana	groEL , hbpE	Dental pulp
Enteric bacteria	uidA , lacZ	Gut contents
Mycobacterium leprae	RLEP, 18-kDa	Bones
Mycobacterium tuberculosis	IS6110	Lung, bone, soft tissue
Treponema pallidum	TPP15	Bone
Yersinia pestis	pla , rpoB	Dental pulp
Ascaris lumbricoides	18S rRNA (176 bp) and cyt B (98 bp)	Coprolites
Enterobius vermicularis	5S rRNA spacer	Coprolites
Plasmodium falciparum	18S rRNA	Bone
Trypanosoma cruzi	Kinetoplast regions	Brain, liver, heart ect.
Anelloviridae species	Whole-genome amplification by cloning and sequencing of Anelloviridae DNA	Dental pulp
Human papilloma virus (HPV-18)	Oligonucleotides HPV 6, 11, 16, 18, 33	Exophytic papillary skin lesion, Condyloma acuminatum
Human T cell lymphotropic virus type 1 (HTLV-1)	HTLV-I-pX (open reading frame encoding p40x, p27x) and HTLV-I-LTR (long terminal repeat)	Mummy bone marrow
Hepatitis B virus	Whole-genome amplification by cloning and sequencing of Hepatitis B virus DNA	Liver