function in individuals with NF1 [62]. These results suggest that the phenotypes in RAS/MAPK syndromes can be ameliorated by the manipulation of RAS/MAPK activity.

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SPECIAL ARTICLE

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Human Variome Project Country Nodes: Documenting Genetic Information within a Country†

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ABSTRACT: The Human Variome Project (http://www.humanvariomeproject.org) is an international effort aiming to systematically collect and share information on all human genetic variation. The two main pillars of this effort are gene/disease-specific databases and a network of Human Variome Project Country Nodes. The latter are nationwide efforts to document the genomic variation

†This article is dedicated to the memory of Professor David Rimoin, the first Chair of the Human Variome Project International Scientific Advisory Committee, whose support and overall contribution for the Human Variome Project was tireless and instrumental for its success.

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reported within a specific population. The development and successful operation of the Human Variome Project Country Nodes are of utmost importance to the success of Human Variome Project's aims and goals because they not only allow the genetic burden of disease to be quantified in different countries, but also provide diagnosticians and researchers access to an up-to-date resource that will assist them in their daily clinical practice and biomedical research, respectively. Here, we report the discussions and recommendations that resulted from the inaugural meeting of the International Confederation of Countries Advisory Council, held on 12th December 2011, during the 2011 Human Variome Project Beijing Meeting. We discuss the steps necessary to maximize the impact of the Country Node effort for developing regional and country-specific clinical genetics resources and summarize a few well-coordinated genetic data collection initiatives that would serve as paradigms for similar projects.
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KEY WORDS: human variome project; country nodes; national/ethnic mutation databases; populations; genomic variation; genomics

Introduction

The Human Variome Project (HVP; http://www.humanvariomeproject.org) is an international initiative to systematically identify and document pathogenic and benign genomic variations worldwide. The project aims to extract, organize, and curate genome variation data from clinical, medical, and research laboratories. The ultimate goal of this project is to improve translational research strategies and clinical decision-making processes. The HVP is a collaborative consortium of internationally renowned scientists and healthcare professionals working on genomics that are organized into working groups around specific topics to produce standards, specify systems requirements, and address related issues [Cotton et al., 2007; Kaput et al., 2009]. The two main avenues of genome variation data collection are (1) gene/disease-specific collection, and (2) country-specific collection.

The HVP Country Nodes (Table 1) are key to the success of the HVP as they would allow: (1) data sharing among diagnostic laboratories and clinics in each country to support nationwide genetic testing services; (2) data archiving in National/Ethnic mutation databases [NEMDBs; Cotton et al., 2009; Patrinos, 2006] to estimate the genetic burden in each country, hence contributing to better targeting of healthcare planning and policy development; and (3) data sharing between NEMDBs and locus-specific databases (LSDBs) or central data repositories (e.g., NCBI and EBI), in a country-specific ethically compliant manner. Building capacity at the national level also provides a necessary platform for engagement across borders. However, there are many issues involved in establishing and, most importantly, running such a Node.

Here, we report the discussions and recommendations that resulted from the inaugural meeting of the International Confederation of Countries Advisory Council (ICCAC), held on 12th December 2011, during the 2011 HVP Beijing Meeting, in which all existing HVP Country Nodes were represented in addition to several human genetics societies (Box 1) to better pursue the HVP aims and goals through the HVP Country Nodes.

Box 1. The Various National Genetics Societies and Regional Human Genetics Networks That Were Represented in the Inaugural Meeting of the ICCAC in Beijing (in Alphabetical Order)

(a) National Genetics Societies
- American Society of Human Genetics
- American College of Medical Genetics
- Austrian Society of Human Genetics
- Belgian Society of Human Genetics
- Hellenic Association of Medical Geneticists
- Spanish Society of Human Genetics

(b) Regional Genetics Networks and Societies
- African Society of Human Genetics
- Centre for Arab Genomic Studies
- European Society of Human Genetics
- Human Genetics Society of Australasia
- International Federation of Human Genetics Societies
- Latin American Network of Human Genetics Societies

Human Variome Project Country Nodes in Practice

At present, 12 HVP Country Nodes are represented in the International Confederation of Countries Council of HVP (Table 1), which have been incorporated in HVP from 2010 and are particularly variable in their stage of development. The outline of establishing an HVP Country Node and the recommendations to collect country-specific genetic data are described elsewhere [Al Aama et al., 2011; Patrinos et al., 2011] and as such will not be discussed here.

The *Australian HVP Node* automatically collects the results of genetic tests performed by Australian Diagnostic laboratories. This genetic data set is stored within a secure repository, which can only be accessed by diagnostics laboratories and medical clinics to assist the diagnosis of patients [Al Aama et al., 2011]. Using this dataset, diagnostics laboratory staff is able to access the cumulative knowledge of every diagnostic laboratory in Australia. The molecular dataset is linked to clinical data housed at hospitals and clinics throughout Australia via the BioGrid service (http://www.biogrid.org.au) and can be accessed by researchers who have obtained approval from a Human Research Ethics Committee.

The *Austrian HVP Node* is at the moment part of the Austrian Human Genetic Society (Österreichische Gesellschaft für Humangenetik, ÖGH). In Austria, there is neither central registration of patient data nor centralized collection of genetic data. As such, the aim of the Austrian HVP Node will be to create a centralized national platform for sharing genetic knowledge and data and providing an easy way to submit variation data directly to international databases, potentially also of interim storage of such data.

The *Belgian HVP Node* is represented by the Belgium Society of Human Genetics, which has Board members from all eight centers offering genetic testing services in the country. The Belgian Plan for Rare Diseases was formulated under the chairmanship of emeritus Professor Jean-Jacques Cassiman. This foresees a National registry, which will be coordinated by the Ministry of Social Affairs and public Health. Presently, there are local disease-specific registries, and some laboratories are entering information into LSDBs. Centers of excellence in the field of cystic fibrosis, neuromuscular disorders, and metabolic diseases do exist and more are foreseen.

Table 1. Existing HVP Country Nodes (as of End of December 2011)

	Node	Node representative	URL	NEMDB[a]
1	Australian	Timothy Smith	http://www.hvpaustralia.org.au	Yes
2	Austrian	Martina Witsch-Baumgartner	http://www.oegh.at	N/A
3	Belgian	Thomy de Ravel	http://www.beshg.be	N/A
4	Chinese	Ming Qi	http://www.genomed.org/lovd	Yes
5	Cypriot	Andreas Hadjisavvas	N/A	N/A
6	Egyptian	Sherifa Ahmed Hamed	http://www.goldenhelix.org	Yes
7	Hellenic	George P. Patrinos	http://www.goldenhelix.org	Yes
8	Kuwaiti	Fahd Al Mulla	http://www.al-mulla.org	Yes
9	Malaysian	Zilafalil bin Alwi	http://1mhgvc.kk.usm.my	N/A
10	Nepalese	Tilak Shreshta	N/A	N/A
11	Spanish	María-Jesús Sobrido	N/A	N/A
12	Vietnamese	Chí Dũng Vũ	N/A	N/A

[a]Existence of a centralized HVP Country Node-specific NEMDB.
N/A, not yet available.

The *Chinese HVP Node* has a central role in the entire project. The Node members have already built several databases of genetic diseases, initially focusing on databases of ion-channel cardiac arrhythmias (LQTS1-12, including KCNQ1, KCNH2, SCN5A, KCNE1, KNCE2, KCNJ2, etc.), *BRCA1* and *BRCA2*, mismatch repair genes (*MMR*), and *APC* genes for breast cancer, Lynch syndrome, and familial adenomatous polyposis (FAP), respectively, in the Chinese population using the Leiden Open Variation Database (LOVD) format [Pan et al., 2011; Zhang et al., 2010; http://www.genomed.org/LOVD]. Data mining was performed by a group of students formed for this purpose using PubMed and some Chinese search engines to collect all the variants in these genes in the Chinese population.

The *Cypriot HVP Node* is in its early phase of development and is under the auspices of the Cyprus Society of Human Genetics. At present, only one institution is performing genetic testing at the diagnostic level, whereas many research laboratories are working on the genetics of rare and common diseases. The Cypriot HVP Node is also in the process of establishing a National Genetic database. Presently, the existence of an ETHNOS-based National Genetic database [Kleanthous et al., 2006; http://www.goldenhelix.org/server/cypriot], which is also expanded along the lines of the MED-GENET European Commission project, is expected to facilitate the establishment of the Cypriot HVP Node National Genetic database.

The *Egyptian HVP Node* is currently run by the Egyptian Neurogenetic Disorders Consortium in Upper Egypt. Egypt has the largest population in the Arab world (about 85 million), with a high percentage (up to 40%) of consanguineous marriages. The Egyptian NEMDB is already available, using the ETHNOS software (http://www.goldenhelix.org/server/egyptian), along the lines of the MED-GENET European Commission project, and can be further expanded to form the basis of the Egyptian HVP Node. Also, the Egyptian National Registry project has been initiated in 2007 for the Genetic Disorders in the National Research Centre, while in the same year the DNA biobank for rare genetic diseases and for subsequent whole genome analysis studies has been established.

The *Hellenic HVP Node was* formally incorporated in the Human Variome Project in 2010. At present, the Hellenic HVP Node is supervised by a board of 11 geneticists from various Greek academic institutions, most of them sitting at the Board of the Hellenic Society of Human Genetics. The Hellenic HVP Node is endorsed by the Hellenic Bioscientists Association (PEV; http://www.pev.gr; content in Greek) and by the Hellenic Society of Human Genetics (http://www.sige.gr; content in Greek) and has been built around the Hellenic National Mutation frequency database, one of the very first NEMDBs developed in 2005 [Patrinos et al., 2005]. The Hellenic NEMDB and the entire Hellenic HVP Node structure are hosted as a contribution to the HVP within the Golden Helix Server of the Golden Helix Institute of Biomedical Research (http://www.goldenhelix.org). The Hellenic NEMDB, such as the Cypriot and the Egyptian NEMDBs, is based on the ETHNOS software [van Baal et al., 2010]. The existence of the Hellenic NEMDB and the subsequent creation of the Hellenic HVP Node has not only promoted the collection of genomic variation in the Hellenic population but has also encouraged new studies to document the genetic heterogeneity of the most common genetic disorders in various parts of the country [Papachatzopoulou et al., 2010; Samara et al., 2007]. Also, board members of the Hellenic HVP Node participate in a nationwide study that have been recently initiated to critically ascertain the general public's awareness and healthcare professionals' opinion on genetics and genetic testing services in Greece [Mai et al., 2011; Pavlidis et al., 2012; Sagia et al., 2011].

The *Kuwaiti HVP Node* was incorporated into the Human Variome Project in 2010 in an attempt to propel local scientists and clinicians into the forefront of genetic research [Ozcelik et al., 2010; Tadmouri et al., 2009]. Currently, the Kuwaiti HVP Node is headed by two members from Kuwait University and the Kuwait Medical Genetic Center (KMGC), who aim at three major objectives: (1) Publicize the importance of HVP and the Kuwaiti HVP Node to the health of the Kuwaiti population and its impact on theranostics. To this end, the Kuwait Node members have recruited collaborators from KMGC Clinical geneticists and senior clinical scientists at the KMGC are now involved in the education of physicians, and the public through seminars and lectures directed toward the importance of the HVP effort. (2) Attract private and public funds for research. The Kuwaiti HVP Node members have initiated an important collaboration with Weill-Cornell Qatar to sequence 360 Arabs from all Gulf States and Lebanon using next generation sequencing technology. Also, the Kuwaiti HVP Node has initiated an ambitious collaboration with the Kuwait Medical Students Association entitled "Adopt a gene project" that encourages groups of medical students to attract private funding from local businesses and sequence a single gene per group and submit the data to the appropriate HVP databases. (3) Submit available data to international and already established databases [Tadmouri et al., 2006]. The National registry contains more than 37,000 files for families in Kuwait with diverse and rare genetic disorders. Transferring this information to international databases and the HVP in an ethically appropriate manner has been initiated by depositing the mutations found in gastrointestinal hereditary tumor syndromes to the INSIGHT database (http://www.insight-group.org).

The *Malaysian HVP Node* is coordinated by the 1Malaysian Human Genome Variation Consortium (1mhgvc), which consists of 60 researchers from 12 Malaysian universities and academic institutions. The research consortium was formed in mid 2010 and received support from the Genetics Society of Malaysia, the Malaysian Society of Human Genetics, the Medical Genetics Society of Malaysia, and the Malaysian Society of Bioinformatics and Computational Biology. Among the objectives of the research consortium is to create the human genome variation map of the major ethnic groups in Malaysia and to study its implications on ethical, legal, and social issues (ELSI); archeogenomics; forensics; and disease genome-wide association studies (GWAS). The Malaysian HVP Node was launched on 9 October, 2010 and during the event, a Malay whole-genome SNP database was launched and has grown in size since then. Currently, a total of 291,718 SNPs from 103 individuals representing the Kelantan, Champa, Banjar, Bugis, Kedah, and Jawa Malay subgroups has been deposited in the database. A mutation database has recently been added to the 1mhgvc databases. Currently, the database contains mutations of the *RB1* and the *MSX1* genes of the Malay patients and these are regularly updated with new mutations and new genes.

The *Spanish HVP Node* started in 2010, with the representative office in the Galician Foundation for Genomic Medicine (Santiago de Compostela) and under the auspices of the Board of the Spanish Society of Human Genetics. The first steps undertaken were directed to awareness raising and gathering of support among the genetics community and other relevant biomedical societies in Spain, mostly in meetings of the Spanish Societies of Neurology, Genetics, and Neuroscience, as well as talks to patient support groups. Also, in the fall of 2010 a document on the HVP was produced and presented to several Health and Science administrations, in which benefits, challenges, and solutions regarding collection of genetic variants by the HVP were described. The document contained also a Node development plan with steps and resources needed, as well as considerations on some legal and ethical aspects, with reference to the HVP recommendations [Povey et al., 2010]. The Regional Government of Galicia approved a two-year grant (2009–2010) to support the collection of genetic variation in neurological and psychiatric disorders in Galicia and, specifically, to contribute to the building of LSDBs and to the HVP. Among the next most important aims of the Spanish HVP Node are to join forces with the ongoing effort of the Spanish Registry for Rare Diseases and to contribute to catalyze the Latin American HVP Node.

The *Vietnamese HVP Node* currently includes only one hospital that has clinical genetics capacity. Also, registries for rare diseases exist in the country that need to be interconnected with the Vietnamese HVP Node. Molecular testing for some monogenic disorders and cancers are available in some centers in the two biggest cities of the country, namely, Hanoi and Ho Chi Minh City.

Finally, the *Nepalese HVP Node* is represented by an eight-member consortium that includes government representatives. There is active interaction with the Chinese HVP Node. To date, there is no established NEMDB in Nepal, although future plans include the development of such database in collaboration with other HVP Nodes that will also include research partnership, technical, and advanced academic training. To this end, there are some initial discussions with the Hellenic HVP Node to provide expertise to these disciplines, as a result of the Beijing International Confederation of Countries Advisory Council meeting. Also, initial funding from HVP coordinating office and the University Grant Commission in Nepal will greatly facilitate these efforts.

Other countries represented in the meeting were South Africa, the United Arab Emirates, Japan, Philippines, the United Kingdom, the United States, Czech Republic, and Finland, while the Latin America region was also represented. Some of these countries have relevant activities that will form the basis of the HVP Nodes. The above illustrates that although the existing HVP Country Nodes are, at present, at various stage of development, they have the potential to make significant contributions to the benefit of local societies, for example, by stratifying molecular diagnostics services, particularly in large heterogeneous populations or by increasing genetics awareness of various stakeholders. Also, the existence of these HVP Country Nodes can motivate the development of other HVP Country Nodes for countries interested to join this international initiative.

Discussion

Over the last decade, various initiatives have been established to capture and archive the genetic heterogeneity in various populations and ethnic groups worldwide (e.g., International HapMap project, 1000 Genomes Project, and Pan-Asian personal genomics initiative). NEMDBs, a large number of which reside in developing countries, aim to extend the effort and fulfil the need to create more comprehensive databases of genetic variants [Patrinos, 2006] by ensuring a thorough documentation of common and rare genetic diseases in each population. For developing countries, a set of recommendations have been recently proposed [Patrinos et al., 2011], that would enable developing countries to better orchestrate the process of capturing genetic variation data linked to pathologies.

One of the stated goals of HVP, as outlined during the inaugural [Melbourne, Australia; Cotton et al., 2007], planning [Costa-Brava, Spain; Kaput et al., 2009], and implementation meetings [Paris, France; Kohonen-Corish et al., 2010] is the realization of the HVP Country Nodes, possibly acting as the locomotive of the entire initiative. However, there are several issues that should be taken into consideration not only while establishing and managing an HVP Country Node but also while coordinating the entire effort. These issues, which have been discussed during the inaugural meeting of the International Confederation of Countries Advisory Council, are outlined below.

Model HVP Country Nodes

There are substantial variations regarding the degree that existing HVP Country Nodes are developed and their activities deployed in the corresponding countries. Despite the fact that most of the HVP Country Nodes have only recently been officially admitted to HVP, some of them, such as the Australian, Chinese, Hellenic, and others have been built around existing structures, such as a NEMDB. However, it has been agreed that at the moment there is no model HVP Country Node because level of development of the Node, genetic, and cultural composition of each country is unique. Also, the International Confederation of Countries Advisory Council noted that the level of development of an HVP Country Node should not be the sole criterion to consider a Country Node as a model because the genetic and cultural composition of each country is unique. An important parameter for assessment of country efforts is whether the HVP Country Node has deployed its activities in concordance with the HVP guidelines and recommendations, as previously described [Al Aama et al., 2011; Patrinos et al., 2011]. Considering the situation in developed countries, such as the United Kingdom, where the genetic services operate in a very organized manner, each HVP Country Node could operate by establishing HVP country-specific councils in various places/cities rather than a Country Node as a whole. This model is consistent with the model adopted by the

European Pharmacogenomics for Every Nation Initiative (PGENI; http://www.pgeni.org) Regional Center with the adoption of PGENI Country Councils, consisting of scientists from the entire country and the number of Council members is largely dependent upon the population size [Mitropoulos et al., 2011]. In any case, defining the "model HVP Country Node" is a challenging goal and this issue can be, at least initially, resolved by establishing a consensus on minimum data content and system requirements, data sharing, and database model standards.

Areas Requiring Standards and Guidelines for Countries

The different areas requiring standards and guidelines for countries were also among the issues discussed during the meeting. The development and management of an HVP Country Node requires the adoption of certain standards, guidelines, and recommendations to assure reliability and validity of the data. An initial set of recommendations have been previously issued, particularly for developing countries [Patrinos et al., 2011], whereas some standards and recommendations already exist for other types of databases (http://www.gen2phen.org), relating to genotype and phenotype object models, mutation database models, LSDB-in-a-box platforms, a minimal content list, a data exchange format, a variation ontology, stable reference sequences for genome regions, and digital identifiers for databases, bioresources, and researchers [Celli et al., 2012; Vihinen et al., 2012; Webb et al., 2011]. There is a need to agree on standards, contents and data models for NEMDBs. This will allow data exchange and integration with other NEMDBs and databases, such as LSDBs, as well as development of software tools. An initial set of recommendations for the ideal content of an NEMDB has been previously suggested [Patrinos, 2006], based on which the *ETHNOS* NEMDB software was developed [van Baal et al., 2010], which is currently being upgraded [Tzimas et al., 2012]. Development of new software in every country would be unnecessary waste of resources and easily lead to large number of isolated databases, which cannot be connected to other resources. ETHNOS could be developed to become such software for NEMDBs. Selecting the latter option is preferred by the attendees of the HVP Council and by some HVP Country Nodes that already use the ETHNOS software for their NEMDBs. The meeting attendees also expressed preference to employ this software for the establishment of NEMDBs in other HVP Country Nodes. The adoption and upgrade of existing software will not only expedite the establishment of new HVP Country Nodes, but will also facilitate the development of existing NEMDBs, by their faster migration to the upgraded software version. We hope a standard will be agreed upon in the near future. Equally important is the assembly and curation of population-specific data collection to ensure that absolute frequencies of disease causing and clinically relevant genome variation are obtained for these populations, particularly those with a high consanguinity rate [Gialluisi et al., 2012].

Software and Systems Between NEMDBs and LSDBs and Strategies for Data Sharing

One of the most critical parameters for the operation of the HVP Country Nodes is the software and systems requirements to accommodate data deposition in each Node and, most importantly, the ability to share and exchange data among different HVP Country Nodes, LSDBs and central repositories. To this end, two main options exist: (1) A gigantic central NEMDB that will be developed for the needs of the HVP that would accommodate all population/ethnic specific datasets, and (2) individual NEMDBs in each HVP Country Node that would be preferably based on the same interoperable Web-enabled platform. The FINDbase database, which was developed for clinically relevant genome variation allele frequencies [http://www.findbase.org; van Baal et al., 2007] may be used as a central unified system [Georgitsi et al., 2011a,b], since the software documents causative mutation and pharmacogenomic marker allele frequencies at a summary level. The interoperable NEMDB approach for data gathering and sharing, which is based on data warehousing principles, has several advantages: (1) databases can be managed and curated from each HVP Country Node representatives, (2) data homogeneity can be ensured since individual NEMDBs will function under the same software, (3) individual population differences, such as social, religious, ethnic differences, can be addressed by minimal software customization without affecting the key functionalities of the main system. Such an approach has already been successfully implemented in the Israeli NEMDB [Zlotogora et al., 2007, 2009], and (4) data gathering can be expedited by assuming a local rather than a central coordinating role. The latter approach has been previously suggested [Patrinos, 2006] and implemented in existing NEMDBs [Georgitsi et al., 2011b; van Baal et al., 2007]. NEMDB database interoperability is also possible not only among NEMDBs that are based on different platforms but also among NEMDBs and other types of databases, for example, LSDBs or central databases. To this end, Café Variome (http://www.cafevariome.org) has been designed to serve as an exchange portal for gene variant (mutation) data. This portal offers users a forum to announce, discover and acquire a comprehensive listing of observed neutral and disease-causing gene variants in patients, unaffected individuals and even populations and, as such, it enables holistic searching across various databases.

In addition to these suggestions and advances, the success of data gathering can be boosted with incentives for data submitters such as microattribution [Giardine et al., 2011] or by encouraging submission of population specific datasets to specialized database journals [Patrinos and Petricoin, 2009] or regulatory incentives [Cotton et al., 2009].

Patient Registries and Other Possible Synergies

Patient registries or clinical genetic databases are equally important aspects of the HVP Country Nodes as summary-level NEMDBs. Patient registries usually contain individual-level data including important genotype–phenotype information that are either maintained locally in hospitals and clinical centers or are big national and some time multicenter supranational initiatives, such as the CFTR2 project (http://www.cftr2.org). However, the establishment and maintenance of patient registries as individual-level clinical genetic databases is a far more demanding task than the development of summary-level NEMDBs. One of the most important challenges is the vital need to ensure patients' anonymity, which dictates removing or safeguarding (e.g., behind firewalls or by encoding) patients' personal information so that they cannot be linked back to their own detailed genotype and phenotype/clinical information. This is particularly important for rare disorders, where one's phenotypic information can reveal his/her identity. This parameter also touches upon the development of databases for clinical trials, which derives from the explosion of individualizing therapeutic interventions. Interconnecting patient registries with mutation databases has been recently discussed [Auerbach et al 2011; Ayme et al 2011], and this combination would be of utmost importance not only for common but also for rare diseases. In actual fact, few HVP Country Nodes, such as the Spanish, the Hellenic, and the Vietnamese HVP

Nodes plan or have already joined forces with the corresponding rare disorders registries.

Listing of Needs

To successfully maintain the existing HVP Country Nodes, to encourage the establishment of new Nodes, and to expand HVP and thereby deliver improved healthcare, additional steps need to be undertaken (summarized in Box 2)

First, a need exists to strengthen the role of the International Confederation of Countries Advisory Council since the Country Nodes will play a vital role in the efforts of the HVP. At the same time, each HVP Country Node and accompanying NEMDB, should bear an electronic seal of HVP as a quality feature, indicating that all necessary recommendations, standards, and guidelines are followed. A dedicated Committee or Working group from the International Confederation of Countries Advisory Council will be responsible to assign this quality feature and monitor the Country Node to ensure it continues to conform to the standards agreed. Reciprocally, the corresponding Human Genetics Societies should formally endorse their Country Node to be admitted to the International Confederation of Countries.

Also, careful consideration and planning of the long-term financial stability and sustainability of each HVP Country Node is an absolute requirement. Viable financial planning by the local HVP Country Node must accompany the country's application and possibly a detailed Strength/Weakness/Opportunities/Threat (SWOT) analysis to allow the HVP Country Node Council to evaluate the feasibility of the proposed approach and the ability of the applicants to successfully establish and manage the Node. Fund raising should be sought locally, such as from governments, national grants, charities, patients' organizations, or others. Similarly, the HVP could also facilitate fund raising efforts by providing grants for the start-up and the initial operation of the Node. Besides grants for start-up of Nodes, funding could also be provided centrally by HVP, for example, to upgrade the NEMDB software, to organize educational and/or outreaching activities, and so on. Funding assistance is particularly important for developing countries.

Consortium members, particularly those from countries having close ties with international organizations like UNESCO and WHO, indicated the importance of these organizations, particularly when speaking to their own governments through Ministries of Health, and Ministries with mandates for science, research, and education. HVP has the status of an international nongovernmental organization in operational relations with UNESCO, which facilitates cooperation with the United Nations. UNESCO has some 56 field offices throughout the world with headquarters in Paris. In addition, UNESCO has a global network of national cooperating bodies known as National Commissions for UNESCO, set up by their respective governments. Presently, there are 197 National Commissions for UNESCO across the world, which operate for the purpose of associating their governmental and nongovernmental bodies in education, sciences, culture, and communication with the work of the Organization. HVP Country Nodes are encouraged to build relationships with their respective UNESCO National Commissions and field offices in areas of UNESCO's mandate. With regard to WHO, as it has a complex structure of approximately 150 country offices, 6 regional offices, and a headquarter in Geneva, building relationships with it are complex. With WHO having a core mandate focusing on public health and its governance being largely in the hands of Ministers of Health, it is important for HVP Country Nodes to develop good relationships with their national Ministries of Health based on how Country Nodes can contribute to the health and well being of the populations they serve.

Media coverage in the national and international arena can add to highlighting the importance of the local HVP effort and improving public knowledge of genetics, genomics, and health. Careful consideration needs to be given to how Country Nodes can best be supported in managing positive relationships with various types of local media. A major aim for the next 12 months is to begin work toward globally agreed standards for databases as well as invite more countries to establish Nodes.

Box 2. Recommendations for Development of HVP Country Nodes

(a) Political measures
- Empowerment of the HVP International Confederation of Countries Advisory Council.
- Develop quality standards and follow their implementation. HVP Country Nodes would have a permission to show HVP seal as a quality feature, to indicate that all necessary recommendations, standards, and guidelines are followed.
- Formal endorsement of each country's application to become an HVP Country Node by the respective national Human Genetics and Genomics Society.
- Agreement on distribution of anonymized information.
- Advocacy with governments: (1) in particular Ministries of Health for awareness of and support for genetic- and genomic-related health in their national health plans; and (2) through Ministries responsible for education, science, and research and through the National Commissions for UNESCO in each country for awareness and support for capacity building in genomics and bioinformatics research and education.

(b) Financial measures
- Careful consideration and planning of a long-term sustainability upon application to become an HVP Country Node.
- Fund raising from local sources, such as national grants, philanthropic organizations, and patients' organizations.
- Financial support by the HVP International Coordinating Office for the start-up and the initial operation of the Country Node, particularly for developing countries.
- Financial support by the HVP Central office for software upgrade and maintenance, for organizing educational and outreaching activities.

(c) Other measures
- Tackling ethical, legal, and societal issues, particularly where patient registries are involved.
- Adopt a country-specific data gathering and sharing approach.
- Provision of education for the general public and healthcare professionals on issues relating to genetics and genomics.
- Development of standards, recommendations, and data models for NEMDBs and encouragement of their implementation to software products.

Conclusions

The success of existing HVP Country Nodes and progress on multiple other planned national platforms underscores the potential value of the HVP Country Node concept. HVP Country Nodes would best be served by coordinating their efforts promoted by the International Confederation of Countries Advisory Council meeting, as described above. A crucial parameter that would guarantee the successful outcome of these efforts is collaborative work of partners from all over the world, which will save time and effort, improve knowledge generation for all, and will result in better local and global solutions for genomic medicine.

Overall, HVP Country Node operational guidelines will help expand the establishment of centers focusing on genome medicine, which will in turn facilitate the fulfillment of the HVP goals toward a comprehensive worldwide genome variation data collection.

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Simple and rapid genetic testing for citrin deficiency by screening 11 prevalent mutations in *SLC25A13*

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ABSTRACT

Citrin deficiency is an autosomal recessive disorder caused by mutations in the *SLC25A13* gene and has two disease outcomes: adult-onset type II citrullinemia and neonatal intrahepatic cholestasis caused by citrin deficiency. The clinical appearance of these diseases is variable, ranging from almost no symptoms to coma, brain edema, and severe liver failure. Genetic testing for *SLC25A13* mutations is essential for the diagnosis of citrin deficiency because chemical diagnoses are prohibitively difficult. Eleven *SLC25A13* mutations account for 95% of the mutant alleles in Japanese patients with citrin deficiency. Therefore, a simple test for these mutations is desirable. We established a 1-hour, closed-tube assay for the 11 *SLC25A13* mutations using real-time PCR. Each mutation site was amplified by PCR followed by a melting-curve analysis with adjacent hybridization probes (HybProbe, Roche). The 11 prevalent mutations were detected in seven PCR reactions. Six reactions were used to detect a single mutation each, and one reaction was used to detect five mutations that are clustered in a 21-bp region in exon 17. To test the reliability, we used this method to genotype blind DNA samples from 50 patients with citrin deficiency. Our results were in complete agreement those obtained using previously established methods. Furthermore, the mutations could be detected without difficulty using dried blood samples collected on filter paper. Therefore, this assay could be used for newborn screening and for facilitating the genetic diagnosis of citrin deficiency, especially in East Asian populations.

1. Introduction

Citrin deficiency is an autosomal recessive disorder that results from mutations in the *SLC25A13* gene [1] and causes two diseases: adult-onset type II citrullinemia (CTLN2; OMIM #603471) and neonatal intrahepatic cholestasis caused by citrin deficiency (NICCD; OMIM#605814) [1–4]. The clinical appearance of these diseases is variable and ranges from almost no symptoms to coma, brain edema, and severe liver failure requiring transplantation [5–8]. In a study of patients with NICCD, only 40% of individuals were identified by newborn screenings to have abnormalities, such as hypergalactosemia, hypermethioninemia, and hyperphenylalaninemia [9]. Other patients were referred to hospitals with suspected neonatal hepatitis or biliary atresia, due to jaundice or discolored stool [9]. Hypercitrullinemia was not observed in all patients [9]. Mutation analysis of *SLC25A13* is indispensable because of the difficulties associated with the chemical diagnosis of citrin deficiency. The *SLC25A13* mutation spectrum in citrin deficiency is heterogeneous, and more than 31 mutations of *SLC25A13* have been identified to date [1,10–18]. However, there are several predominant mutations in patients from East Asia. As shown in Table 1, 6 prevalent mutations account for 91% of the mutant alleles in the Japanese population [12,19]. Five additional mutations also occur within a 21-bp cluster in exon 17 (Table 1 and Fig. 1D). The six prevalent mutations, together with the five mutations in exon 17, account for 95% of the mutant alleles in Japan [12,19].

Several different methods, such as direct sequencing, PCR restriction fragment length polymorphism (PCR-RFLP), and denaturing high performance liquid chromatography (DHPLC), are currently used for the detection of mutations in *SLC25A13* [1,10–14,19]. However, these methods are too complex for clinical use. Direct sequencing is a standard but cumbersome method. The PCR-RFLP method is

Abbreviations: CTLN2, adult-onset type II citrullinemia; FRET, fluorescence resonance energy transfer; HRM, high resolution melting; NICCD, neonatal intrahepatic cholestasis caused by citrin deficiency; Tm, melting temperature.

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Table 1
Seven primer/probe sets and 11 targeted mutations of *SLC25A13*.

Primer/probe set	Mutation		Location	Nucleotide change	Effects of mutations	Allele frequency*[19]	References
A	Mutation [I]	:851del4	exon 9	c.851_854delGTAT	p.R284fs(286X)	33.2%	[1]
B	Mutation [II]	:g.IVS11+1G>A	intron 11	c.1019_1177del	p.340_392del	37.6%	[1]
C	Mutation [III]	:1638ins23	exon 16	c.1638_1660dup	p.A554fs(570X)	3.4%	[1]
D	Mutation [IV]	:S225X	exon 7	c.675C>A	p.S225X	5.3%	[1]
E	Mutation [V]	:g.IVS13+1G>A	intron 13	c.1231_1311del	p.411_437del	8.2%	[1]
F	Mutation [XIX]	:IVS16ins3kb	intron 16	c. aberrant RNA	p.A584fs(585X)	4.6%	[19]
G	Mutation [VI]	:1800ins1	exon 17	c.1799_1800insA	p.Y600X	1.3%	[10]
	Mutation [VII]	:R605X	exon 17	c.1813C>T	p.R605X	0.90%	[10]
	Mutation [VIII]	:E601X	exon 17	c.1801G>T	p.E601X	1.2%	[11]
	Mutation [IX]	:E601K	exon 17	c.1801G>A	p.E601K	0.30%	[11]
	Mutation [XXI]	:L598R	exon 17	c.1793T>G	p.L598R	0%	[15]
						Total 95.1%	

* The frequency of each mutant allele among Japanese patients with citrin deficiency.

complicated and can lead to genotyping errors, due to incomplete digestion by the restriction enzymes. DHPLC is time-consuming and requires expensive equipment. Thus, there is a strong need for the development of a simple test for these mutations.

The goal of this study was to establish a rapid and simple test for the detection of the 11 most common *SLC25A13* mutations. We adopted the HybProbe format (Roche) for the detection of the mutations using real-time PCR followed by a melting-curve analysis with adjacent hybridization probes [20,21]. This assay can be completed in less than 1 h and has the advantage of being a closed-tube assay. The fundamental process for detecting point mutations using the HybProbe assay is presented in Fig. 1A. The 11 prevalent mutations contain not only point mutations but also include a 4-bp deletion and insertions of 1-bp, 23-bp and 3-kb genomic fragments (Table 1 and Fig. 1). Careful design of the PCR primers and HybProbes enabled us to test for these various *SLC25A13* mutations.

2. Methods

2.1. Subjects

CTLN2 and NICCD were diagnosed, as previously described [9,10,19,22–24]. Genomic DNA of the patients was obtained from peripheral blood leukocytes using the DNeasy blood kit (Qiagen Inc., Valencia, CA, USA). Genomic DNA was purified from filter paper blood samples using the ReadyAmp Genomic DNA Purification System (Promega, Madison, WI, USA). Mutations in these DNA samples were analyzed at Kagoshima University using a combination of PCR with or without restriction enzyme digestion or by direct sequencing, as previously described [1,10–14,19]. Another set of samples was obtained from 420 healthy volunteers (mainly from Miyagi prefecture in the northeastern region of Japan) at Tohoku University. Genomic DNA from leukocytes was extracted, as described above.

2.2. Detection of seven prevalent mutations in SLC25A13 using the HybProbe assay

HybProbe probes comprise a pair of donor and acceptor oligonucleotide probes designed to hybridize adjacent to their target sites in an amplified DNA fragment [20,21]. The donor probes are labeled at their 3′ end with fluorescein isothiocyanate (FITC), whereas the acceptor probes are labeled at their 5′ end with LC Red640; these acceptor probes are phosphorylated at their 3′ end to prevent extension by the DNA polymerase. When two probes hybridize to the amplicon, the fluorescent dyes are located within 5 bases of each other, which allows fluorescence resonance energy transfer (FRET) between the excited FITC and the LC Red640; this process emits light that can be quantified by real-time PCR. Following PCR amplification, a melting-peak analysis is performed. The melting peak is produced by the reporter probe, which has a lower melting temperature (Tm) than the other probe, called the anchor probe. As the reporter melts from the target, the fluorophores are separated, and the FRET ceases. The Tm of the reporter probe determines the reaction

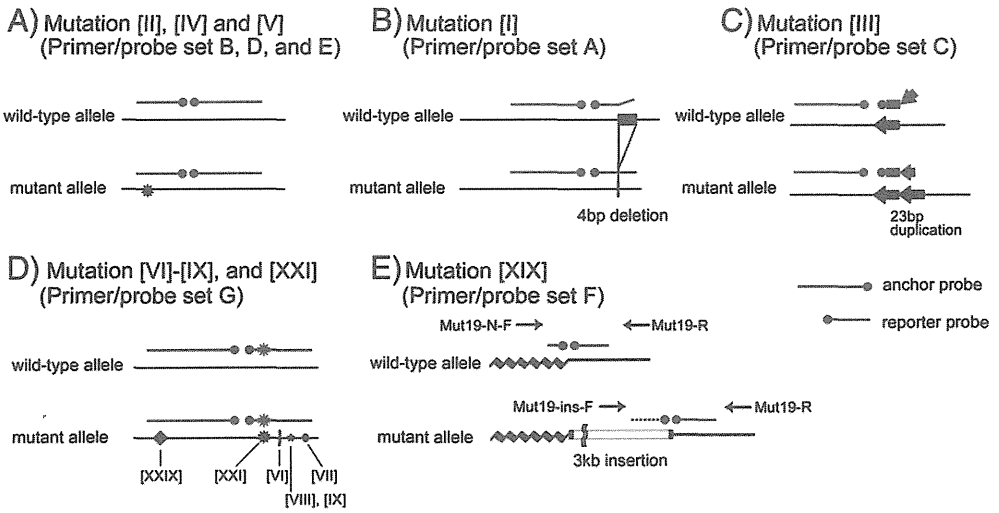

Fig. 1. Principle of *SLC25A13* mutation detection by melting-curve analysis with the HybProbe assay. In primer/probe sets A–E, and G, PCR was performed with a pair of primers, whereas in primer/probe set F, two forward primers and one common reverse primer were used for the amplification of both wild-type and mutant alleles. Note that mutation [XXIX], located on the anchor probe of primer/probe set G, is a non-target mutation.

specificity (i.e., binding of the probe to a perfectly matched sequence rather than to regions with sequence mismatches).

Seven primer/probe sets were designed for this study. Fig. 1 shows a schematic diagram of the strategy for mutation detection using these primer/probe sets. Tables 1 and 2 list the primer/probe sets and corresponding sequences and primer concentrations that were used to target the 11 mutations. Primer/probe sets A, B, C, D, E, and F were designed to detect mutations [I], [II], [III], [IV], [V], and [XIX], respectively. Primer/probe set G was designed to detect the five mutations clustered on exon 17: mutations [VI], [VII], [VIII], [IX], and [XXI] (Fig. 1D). All primers and probes were synthesized based on the NCBI reference SLC25A13 gene sequence (GenBank accession no. NM_014251) with the exception of mutation [XIX]:IVS16ins3kb, which was designed according to [19].

Real-time PCR and subsequent melting curve analyses were performed in a closed tube using a 20-μL mixture on a LightCycler 1.5 (Roche Diagnostics, Tokyo, Japan). The PCR mixture contained 2.0 μL of genomic DNA (10–50 ng), 0.5 μM of forward primer, 0.5 or 0.1 μM of reverse primer, 0.2 μM of each sensor and anchor probe, and 10 μL of Premix ExTaq™ (Perfect Real Time) reagent (TaKaRa Bio Inc., Otsu, Japan).

The thermal profile conditions were identical for all seven assays and consisted of an initial denaturation step (30 s at 95 °C), followed by 45 amplification cycles with the following conditions: denaturation for 5 s at 95 °C and annealing and extension for 20 s at 60 °C. The transition rate between all steps was 20 °C/s. After amplification, the samples were held at 37 °C for 1 min, followed by the melting curve acquisition at a ramp rate of 0.15 °C/s extending to 80 °C with continuous fluorescence acquisition.

Table 2
Primers, probes and target amplicon sequences, target mutation sites, and primer concentrations.

Primer/probe set	Name	Sequences of PCR products, primer locations, probe sequences, and mutation sites (5′ to 3′)	Concentration (μmol/L)
A		GGCTATACTGAAATATGAGAAatgaaaaaagggatgtttttaaatttttataatgtaaattgtaataaattggtatatttgttgcttgtgtttgtttttcccctacagac	
		gtatgaccttagcagacattgaacggattgctcctctggaagagggaactctgccCTTTAACTTGGCTGAGG (181 bp)	
	Mut1-F	GGCTATACTGAAATATGAGAA	0.5
	Mut1-R	CCTCAGCCAAGTTAAAG	0.5
	Mut1-UP	ATGTAAATTGTAATAAATTGGTATATTTGTTGCTTGTGTT-FITC	
	Mut1-DW	LC Red640-GTTTTTCCCCTACAGAC**GACC**-P	
B		GAATGCAGAACCAACGAtcaactggctcttttgtgggagaactcatgtataaaaacagctttgactgttttaagaaagtgctacgctatgaaggcttctt	
		tggactgtatagag**g**ttagtgccacatgctcaatacctgttaggtgaaataacactcaaaggtttggtttctcatcttagtgcctGACATGAATTAGCAAGACTG (205 bp)	
	Mut2-F	GAATGCAGAACCAACGA	0.5
	Mut2-R	CAGTCTTGCTAATTCATGTC	0.1
	Mut2-UP	ACCTAACAGGTATTGAGCATGTG-FITC	
	MUt2-DW	LC Red640-CACTAA**C**CTCTATACAGTCCA-P	
C		GCAGTTCAAAGCACAGTTATTtttatatagtgagaatgtgaccagactgagatggtgttgtgtctctcctgcaggtatgcctgcagcatctttagtg	
		accccctgctgatgttatcaagacgagattacaggtg	
		gctgcccggg(**gagatta**caggtggctgcccggg)ctggccaaaccaCTTACAGCGGAGTGATAGAC (175 bp)	
	Mut3-F	GCAGTTCAAAGCACAGTTATT	0.5
	Mut3-R	GTCTATCACTCCGCTGTAAG	0.5
	Mut3-UP	ACCCCTGCTGATGTTATCAAGACGAGATTACAGGT-FITC	
	Mut3-DW	LC Red640-GCTGCCCGGG**GAGATTA**-P	
D		TCAATTTATTTGAGGCTGCtggaggtaccacatcccatcaagttagtttctcctattttaatggatttaattcgctccttaacaac	
		atggaactcattagaaagatctatagcactc	
		tggctggcaccaggaaagatgttgaagtGACTAAGGGTGAGTGAGAA (164 bp)	
	Mut4-F	TCAATTTATTTGAGGCTGC	0.5
	Mut4-R	TTCTCACTCACCCTTAGTC	0.5
	Mut4-UP	AATGGATTTAATT**C**GCTCCTTAACA-FITC	
	Mut4-DW	LC Red640-ATGGAACTCATTAGAAAGATCTATAGCACTC-P	
E		TGCACAAAGATGGTTCGgtcccacttgcagcagaaattcttgctggaggctgc**g**taagtacctttgaagctctcttcattgaaaagacttgtttcac	
		atatatatcactaccatggtcaacaggtgtggactaaggcttctgttTAACCACAGATCCTGCA (162 bp)	
	Mut5-F	TGCACAAAGATGGTTCG	0.5
	Mut5-R	TGCAGGATCTGTGGTTA	0.5
	Mut5-UP	GTGAAACAAGTCTTTTCAATGAAGAGAGCTTC-FITC	
	Mut5-DW	LC Red640-AAGGTACTTA**C**GCAGCCTC-P	
F	normal allele	GGAGCTGGTGGTATGGAAatgtgttcttaactaactctttggtatcaggtaaatttttaaaatatctaattatatctgtgatttctc	
		catttttttaaagctcgtgtatttcgatcctcaccccagtttggt	
		gtaactttgctgacttacgaattgctacagcgatggttctacattgattttggaggagtgtaagtatcatgctaaatctgctgctaaatttt	
		GGCTGCTGCTAATGCTC (244 bp)	
	insertion allele	CCATCTTCCTCCTCCCTTggcagccccgccccccgatttctccatttttttaaagctcgtgtatttcgatcctcaccccagtttggt	
		gtaactttgctgacttacgaattgctacagcgatggttctacattgatttt	
		ggaggagtgtaagtatcatgctaaatctgctgctaaattttGGCTGCTGCTAATGCTC (196 bp)	
	Mut19-N-F	GGAGCTGGTGGTATGGAA	0.5
	Mut19-ins-F	CCATCTTCCTCCTCCCTT	0.5
	Mut19-R	GAGCATTAGCAGCAGCC	0.5
	Mut19-UP	ACCAAACTGGGGTGAGGATCGAAATACACGAGCTTTAAAAAAATG-FITC	
	Mut19-N-DW	LC Red640-AGAAATCACAGATATAATTAGATATTT-P	
	Mut19-ins-DW	LC Red640-AGAAATCGGGGGGCGGGG-P	
G		TCTTAACTAACTCTTTGGTATCAGGTaaatttttaaaatatctaattatatctgtgatttctccatttttttaaagctcg	
		tgtatttcgatcctcaccccagtttggtgtaactttgc**t**gactt**a(a)cg**aattgctacag**c**ga	
		tggttctacattgattttggaggagtgtaagtatcatgctaaatctgctgctaaattttGGCTGCTGCTAATGCTC (217 bp)	
	Mut6-9, 21-F	TCTTAACTAACTCTTTGGTATCAGGT	0.5
	Mut6-9, 21-R	GAGCATTAGCAGCAGCC	0.5
	Mut6-9, 21-UP	TGTATTTCGATCCTCACCCCAGTTTGGTGTAACTT-FITC	
	Mut6-9, 21-DW	LC Red640-GC**G**GACTT**ACG**AATTGCTACAG**C**GA-P	

Upper case and underlined letters indicate the locations of primers and probes, respectively. Inserted DNA is shown in parenthesis. Nucleotides in boldface were used for mutation detection.
F: forward, R: reverse, UP: upstream, DW: downstream, N: normal allele, ins: insertion allele, FITC: fluorescein isothiocyanate, P: phosphate.

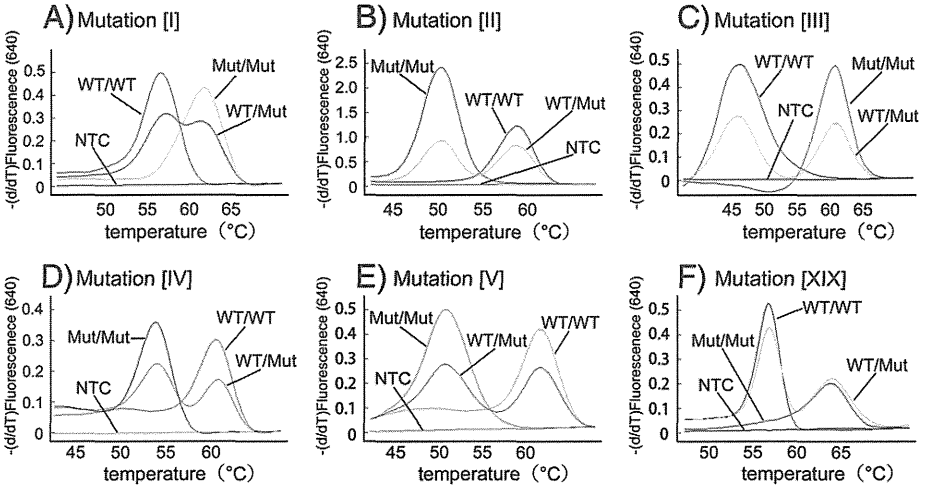

Fig. 2. Typical melting curves used in the detection of mutations [I–V] and [XIX]. Each assay using primer/probe sets A–F is displayed in a separate graph (A–F). WT: wild-type allele, Mut: mutant allele, NTC: no DNA template control.

2.3. Validation of the mutation detection system

After establishing the protocol for detecting the 11 prevalent mutations, 50 DNA samples from patients' blood were sent from Kagoshima University to Tohoku University for the validation of this system in a single-blind manner. Similarly, 26 DNA samples purified from paper-filter blood samples were analyzed in the same manner as the blood DNA samples.

2.4. Estimation of the carrier frequency

For the estimation of the heterozygous carrier frequency, 420 genomic DNA samples from healthy volunteers were screened using the HybProbe analysis for the 11 prevalent mutations. All detected mutations were confirmed by direct sequencing.

2.5. Ethics

This study was approved by the Ethical Committees of Tohoku University School of Medicine and Kagoshima University. Written informed consent was obtained from all participants or their guardians.

3. Results

3.1. Development of the mutation detection system

In primer/probe sets B, D, and E, the reporter probes were designed to be complimentary to the wild-type allele (Fig. 1A). To allow for an improved detection of the mutations, primer/probe sets A and C were designed to be complementary to the mutant allele (Figs. 1B, C). In the primer/probe set F, two forward PCR primers, which were specific to the wild-type and the mutant alleles, were used with a common reverse primer for the co-amplification of the wild-type and 3-kb insertion alleles (Fig. 1E). Two reporter probes, which had a common anchor probe, were used for the detection of the wild-type and mutant alleles. Because the two reporter probes had different melting temperatures, we were able to identify the allele that was amplified. Fig. 2 shows representative results of the melting curve analyses using the primer/probe sets A–F, in which all of the mutant alleles generated distinct peaks corresponding to the wild-type alleles.

In the primer/probe set G, we used a reporter probe that was complementary to the mutant [XXI] allele (Fig. 1D). All five mutations in exon 17 were successfully differentiated from the wild-type allele (Figs. 3A–E). The [XXIX] mutation is an additional mutation in exon

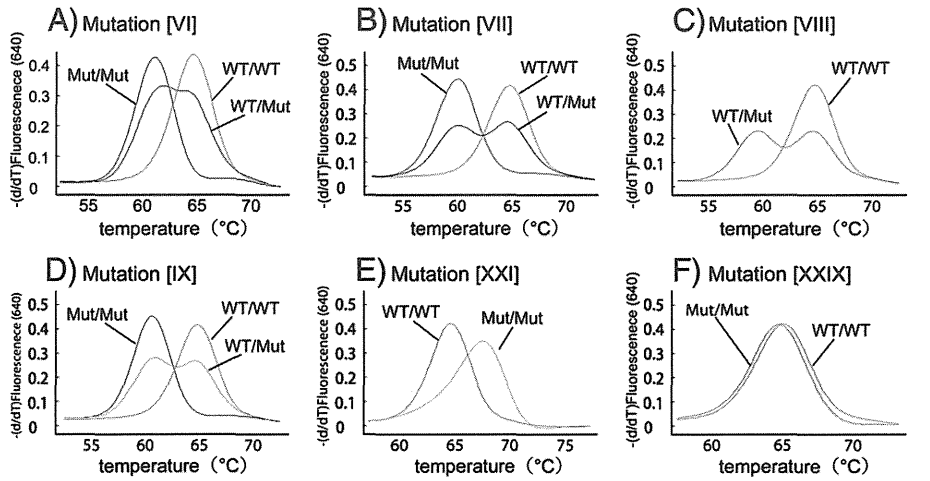

Fig. 3. Typical melting curves used in the detection of mutations [VI–XI], [XXI], and [XXIX] on exon 17. Genotyping was performed using primer/probe set G. Each melting curve for a target mutation is displayed in a separate graph (A–F). Note that mutation [XXIX] (F) is a non-target mutation on the anchor probe. WT: wild-type allele, Mut: mutant allele.

17 that is not listed in Table 1. The [XXIX] mutation is located in the anchor-probe binding site and not on the reporter-probe binding site (Fig. 1D). To examine the effect of mutations on the anchor probe, we genotyped a patient with a heterozygous [XXIX] mutation using primer/probe set G (Fig. 3F). We found no change in the melting curves between the wild-type allele and the [XXIX] allele, thereby suggesting that point mutations within the anchor probe sequence have little effect on the melting curve analysis.

3.2. Validation

The genotypes determined at Tohoku University using the proposed method and those determined at Kagoshima University using a previously published method were identical for the 11 common mutations (Table S1 in supplementary material). We performed a similar test using DNA samples purified from filter-paper blood samples to determine if this method could be used for newborn screening. The genotypes determined in both laboratories were identical for all 26 DNA samples (Table S2 in supplementary material).

3.3. Frequency of eleven prevalent mutations

We found four heterozygous carriers of mutation [I], three of mutation [II], and two of mutation [V]. In addition, primer/probe set G detected one heterozygous mutation, which was confirmed as mutation [VIII] by direct sequencing. Altogether, 10 mutations were detected in 420 Japanese healthy controls.

4. Discussion

We developed a simple and rapid genetic test using real-time PCR combined with the HybProbe system for the 11 prevalent mutations in *SLC25A13*: mutations [I], [II], [III], [IV], [V], [VI], [VII], [VIII], [IX], [XIX], and [XXI]. This genetic test is a closed-tube assay in which no post-PCR handling of the samples is required. In addition, the genotyping is completed within 1 h. This test can utilize DNA samples purified from both peripheral blood and filter-paper blood. The reliability of the test was confirmed by genotyping 76 blind DNA samples from patients with citrin deficiency, including 50 peripheral blood and 26 filter-paper blood DNA samples. Because screening for the 11 targeted mutations would identify 95% of mutant alleles in the Japanese population [19], both, one, and no mutant alleles are expected to be identified in 90.4%, 9.3%, and less than 0.3% of patients, respectively. This genetic test would be useful not only in Japan but also other East Asian countries, including China, Korea, Taiwan and Vietnam, in which the same mutations are prevalent. Our test is expected to detect 76–87% of the mutant alleles in the Chinese population [12,19,25], 95–100% in the Korean population [12,19,26], 60–68% in the Taiwanese population [27,28], and 100% in the Vietnamese population [12,19]. If we were to prepare a primer/probe set for mutation [X]:g.IVS6+5G>A [12], which is prevalent in Taiwan, the estimated sensitivity would exceed 90% in the Taiwanese population [27,28].

Recently, the high resolution melting (HRM) method was reported to be suitable for the screening of mutations in the diagnosis of citrin deficiency [28]. HRM analysis is a closed-tube assay that screens for any base changes in the amplicons. The presence of SNPs anywhere on the amplicons can affect the melting curve, thereby suggesting that HRM is not suitable for screening for known mutations, but rather, is best suited to screening for unknown mutations. When we detected one heterozygous prevalent mutation, we performed HRM screening for all 17 exons of *SLC25A13*. After HRM screening, only the HRM-positive exons were subjected to direct sequencing analysis. Several mutant alleles were identified using this approach.

The frequency of homozygotes, including compound heterozygotes, presenting *SLC25A13* mutations in the population at Kagoshima (a prefecture in the southern part of Japan) has been calculated to be 1/17,000 based on the carrier rate (1/65) [19]. The prevalence of NICCD has been also reported to be 1/17,000–34,000 [29]. In this study, the carrier rate in Miyagi (a prefecture in northern Japan) was 1/42 (95% confidential interval, 1/108–1/26), thereby yielding an estimated frequency of patients with citrin deficiency of 1/7,100. Our result, together with the previous report [19], suggests that a substantial fraction of the homozygotes or compound heterozygotes of SLC25A13 mutations was asymptomatic during the neonatal period.

The early and definitive diagnosis of citrin deficiency may be beneficial for patients with citrin deficiency by encouraging specific dietary habits and avoiding iatrogenic worsening of brain edema by glycerol infusion when patients develop encephalopathy [30,31]. Because the screening of blood citrulline levels by tandem mass analysis at birth does not detect all patients with citrin deficiency, the development of a genetic test would be welcomed. In this study, we demonstrated that genomic DNA extracted from filter paper blood samples was correctly genotyped, thereby indicating the feasibility of newborn screening using this genetic test. If 100,000 babies in the northern part of Japan were screened by this method, we would detect 14 homozygotes or compound heterozygotes with *SLC25A13* mutations and 2400 heterozygous carriers. In 2400 heterozygous carriers, we would expect to observe only 1 to 2 compound heterozygotes with one target and one non-target mutation. The estimated frequency of babies with two non-target mutations is 0.04/100,000. Our genetic method would therefore allow us to screen newborn babies efficiently. If we performed this genetic test in a high-throughput real-time PCR system, such as a 384- or 1,536-well format, the cost per sample could be lowered.

In conclusion, we have established a rapid and simple detection system using the HybProbe assay for the 11 prevalent mutations in *SLC25A13*. This system could be used to screen newborns for citrin deficiency and may facilitate the genetic diagnosis of citrin deficiency, especially in East Asian populations.

Supplementary materials related to this article can be found online at doi:10.1016/j.ymgme.2011.12.024.

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RESEARCH ARTICLE

AMERICAN JOURNAL OF medical genetics PART A

Prevalence and Clinical Features of Costello Syndrome and Cardio-Facio-Cutaneous Syndrome in Japan: Findings From a Nationwide Epidemiological Survey

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Costello syndrome and cardio-facio-cutaneous (CFC) syndrome are congenital anomaly syndromes characterized by a distinctive facial appearance, heart defects, and intellectual disability. Germline mutations in *HRAS* cause Costello syndrome, and mutations in *KRAS*, *BRAF*, and *MAP2K1/2* (MEK1/2) cause CFC syndrome. Since the discovery of the causative genes, approximately 150 new patients with each syndrome have been reported. However, the clinico-epidemiological features of these disorders remain to be identified. In order to assess the prevalence, natural history, prognosis, and tumor incidence associated with these diseases, we conducted a nationwide prevalence study of patients with Costello and CFC syndromes in Japan. Based on the result of our survey, we estimated a total number of patients with either Costello syndrome or CFC syndrome in Japan of 99 (95% confidence interval, 77–120) and 157 (95% confidence interval, 86–229), respectively. The prevalences of Costello and CFC syndromes are estimated to be 1 in 1,290,000 and 1 in 810,000 individuals, respectively. An evaluation of 15 adult patients 18–32 years of age revealed that 12 had moderate to severe intellectual disability and most live at home without constant medical care. These results suggested that the number of adult patients is likely underestimated and our results represent a minimum prevalence. This is the first epidemiological study of Costello syndrome and CFC syndrome. Identifying patients older than 32 years of age and following up on the patients reported here is important to estimate the precise prevalence and the natural history of these disorders.

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INTRODUCTION

Costello syndrome (OMIM 218040), a rare, multiple congenital anomaly syndrome, was first described by Costello in 1971 [Costello, 1971]. Costello syndrome is characterized by intellectual disability, a high birth weight, neonatal feeding problems, short stature, congenital heart defects, curly hair, distinctive facial features, nasal papillomata, and loose integuments of the back of the hands [Hennekam, 2003]. Cardio-facio-cutaneous (CFC) syndrome (OMIM 115150) was first described in 1986 [Reynolds et al., 1986]. Affected individuals present with heart defects, short stature, frequent intellectual disability, and ectodermal abnormalities such as sparse, fragile hair, hyperkeratotic skin lesions, and a generalized ichthyosis-like condition. These syndromes overlap phenotypically with Noonan syndrome (OMIM 163950). We discovered that *HRAS* mutations of are causative of Costello syndrome [Aoki et al., 2005], and we and other group subsequently identified mutations in *KRAS*, *BRAF*, and *MAP2K1/2* (MEK1/2) in patients with CFC syndrome [Niihori et al., 2006; Rodriguez-Viciana et al., 2006]. Missense mutations in *PTPN11*, *SOS1*, *KRAS*, *RAF1*, and *NRAS* have been identified in individuals affected by Noonan syndrome or Noonan syndrome with multiple lentigines, previously known as LEOPARD syndrome (OMIM 151100, 611554) [Tartaglia et al., 2001; Schubbert et al., 2006; Pandit et al., 2007; Razzaque et al., 2007; Roberts et al., 2007; Tartaglia et al., 2007; Cirstea et al., 2010]. Mutations in *SHOC2* have been identified in patients with Noonan-like disorder with loose anagen hair (OMIM 613563) [Cordeddu et al., 2009]. Because the clinical manifestations of these diseases are similar, a novel disease entity was proposed that consists of a syndrome characterized by a dysregulation of the RAS/MAPK signaling pathway [Aoki et al., 2008; Tidyman and Rauen, 2009].

Evaluation of the clinical manifestations of Costello and CFC syndromes revealed the similarities and differences between individuals with the diseases. Individuals with either syndrome have distinctive facial features; full cheeks and a large nose and mouth are characteristic of individuals with Costello syndrome, and a high cranial vault, bitemporal narrowing and a hypoplastic supraorbital ridge are characteristic of individuals with CFC syndrome. Wrinkled palms and soles have been thought to be characteristic features of individuals with Costello syndrome. A recent evaluation showed that 30% of individuals with CFC syndrome also have wrinkled palms and soles [Narumi et al., 2007]. Heart defects have been frequently reported in individuals with Costello and CFC syndromes; 61% of patients with Costello syndrome have hypertrophic cardiomyopathy, while 44 and 56% of Costello syndrome patients have congenital heart defects and arrhythmia, respectively. In contrast, hypertrophic cardiomyopathy, congenital heart defects, and arrhythmia have been observed in 36, 45, and 9%, respectively, of patients with CFC syndrome [Lin et al., 2011].

Approximately 10–15% of individuals with Costello syndrome develop malignant tumors, including transitional carcinomas in the bladder, rhabdomyosarcomas, and neuroblastomas [Aoki et al., 2008; Kratz et al., 2011]. Although association of malignant tumors has been rarely reported in individuals with CFC syndrome, we observed patients with *BRAF* mutations who developed acute lymphoblastic leukemia (ALL) and non-Hodgkin lymphoma [Niihori et al., 2006; Makita et al., 2007; Ohtake et al., 2011].

The number of patients known to have these diseases is growing due to the identification of the causative genes. At least 150 genotyped patients with Costello syndrome have been reported [Lin et al., 2011]. In addition, more than 100 individuals with CFC syndrome have been reported in the literature [Rauen, 2007]. Till date, however, an epidemiological study has not been conducted. In order to identify the precise number of patients with these diseases, the natural history of the diseases, the prognosis and the rate of tumor development, we performed a nationwide investigation of both Costello syndrome and CFC syndrome.

MATERIALS AND METHODS

First-Stage Survey

The protocol we followed was established by the Research Committee on the Epidemiology of Intractable Diseases funded by the Ministry of Health, Labour and Welfare of Japan [Kawamura et al., 2006]. The prevalence of intractable diseases, including moyamoya disease, pancreatitis and sudden deafness, were all reported using this protocol [Teranishi et al., 2007; Kuriyama et al., 2008; Satoh et al., 2011]. The protocol consists of a two-stage postal survey. The first-stage survey aimed to estimate the number of individuals with Costello syndrome or CFC syndrome, and the second-stage survey aimed to identify the clinico-epidemiological features of the two syndromes.

The pediatric departments of all hospitals were identified based on a listing of hospitals as of 2008 supplied by the R & D Co.LTD (Nagoya, Japan). These hospitals were classified into seven categories according to the type of institution (i.e., university hospital or general hospital) and the number of hospital beds. Hospitals were then randomly selected from each of these categories for sampling. The sampling rate was approximately 5, 10, 20, 40, 80, and 100% of general hospitals with less than 100 beds, 100–199 beds, 200–299 beds, 300–399 beds, 400–499 beds, and 500 or more beds, respectively, and 100% of university hospitals [Kuriyama et al., 2008]. To increase the efficiency of the study, we sent a survey form to 205 pediatricians and 44 clinical geneticists working in the departments of gynecology, genetics, or ophthalmology in university hospitals (See Supplemental eTable I in supporting information online). We also selected 29 physicians who previously sent patient samples to our facility for molecular analysis. These hospitals were separately classified into a "selected hospitals" category, and all hospitals in this category were surveyed. Another 205 institutions that treat the disabled were included in order to identify adult patients.

The survey was mailed out to the targeted departments of health institutes in October 2009 along with cover letters. A simple questionnaire was used to ask about the number of patients with Costello syndrome known to have an *HRAS* mutation, CFC syndrome patients with mutations in *KRAS*, *BRAF*, or *MAP2K1/2*

(*MEK1/2*) and clinically suspected patients. Photographs of patients, obtained with their specific consent, were printed on the brochure describing the disease overview. In December 2009, a second request was sent to departments that had not responded by the earlier deadline (the end of November 2009). Following the first-stage survey, we sent acknowledgement letters to departments that had responded.

Genetic Testing of Clinically Suspected Patients

Blood samples from 42 individuals clinically suspected to have Costello or CFC syndrome were sent to our facility. After DNA was extracted by a standard protocol, we performed genetic screening for all four exons of *HRAS* and 14 exons of *BRAF*, *MAP2K1*, *MAP2K2*, and *KRAS* in which mutations have been previously identified (*BRAF* exons 6 and 11–16, *MAP2K1* exons 2 and 3, *MAP2K2* exons 2 and 3 and *KRAS* exons 1, 2, and 5) (Fig. 1). In samples negative for the first screening, we further analyzed all of the known causative genes for Noonan syndrome and related disorders (including the remaining exons in *BRAF*, *KRAS*, *MAP2K1*, and *MAP2K2*, all 17 exons in *RAF1*, all 23 exons in *SOS1*, all 4 exons in *NRAS*, and exon 1 of *SHOC2*). The clinical manifestations of the patients were evaluated by clinical dysmorphologists (K.K., H.O., H.K., N.O., S.M.).

Second-Stage Survey

The second questionnaires were forwarded to the departments that reported patients with Costello or CFC syndrome on the first questionnaires. Detailed clinical information was collected, including the age, gender, growth and development pattern, cardiac defects, central nervous system defects, craniofacial characteristics, musculoskeletal characteristics, skin characteristics, tumors, identified mutations, and the facility where the genetic analysis had been performed. Duplicate results were excluded using the information regarding the patient's age, gender, and the type of mutations, if available. The Ethics Committee of Tohoku University School of Medicine approved this study. We obtained informed consent from all subjects involved in the genetic testing and specific consent for the photographs from three patients shown in Figure 1.

Estimation of Prevalence

We first estimated the number of patients in departments who responded the first survey, using the number of mutation-positive patients from the first-stage postal survey and the number of newly identified patients by mutational analysis in the current study. PR_k denotes the number of mutation-positive patients reported in the first-stage survey. The estimate was made based on the assumption that mutation-positive patients equally existed in the clinically

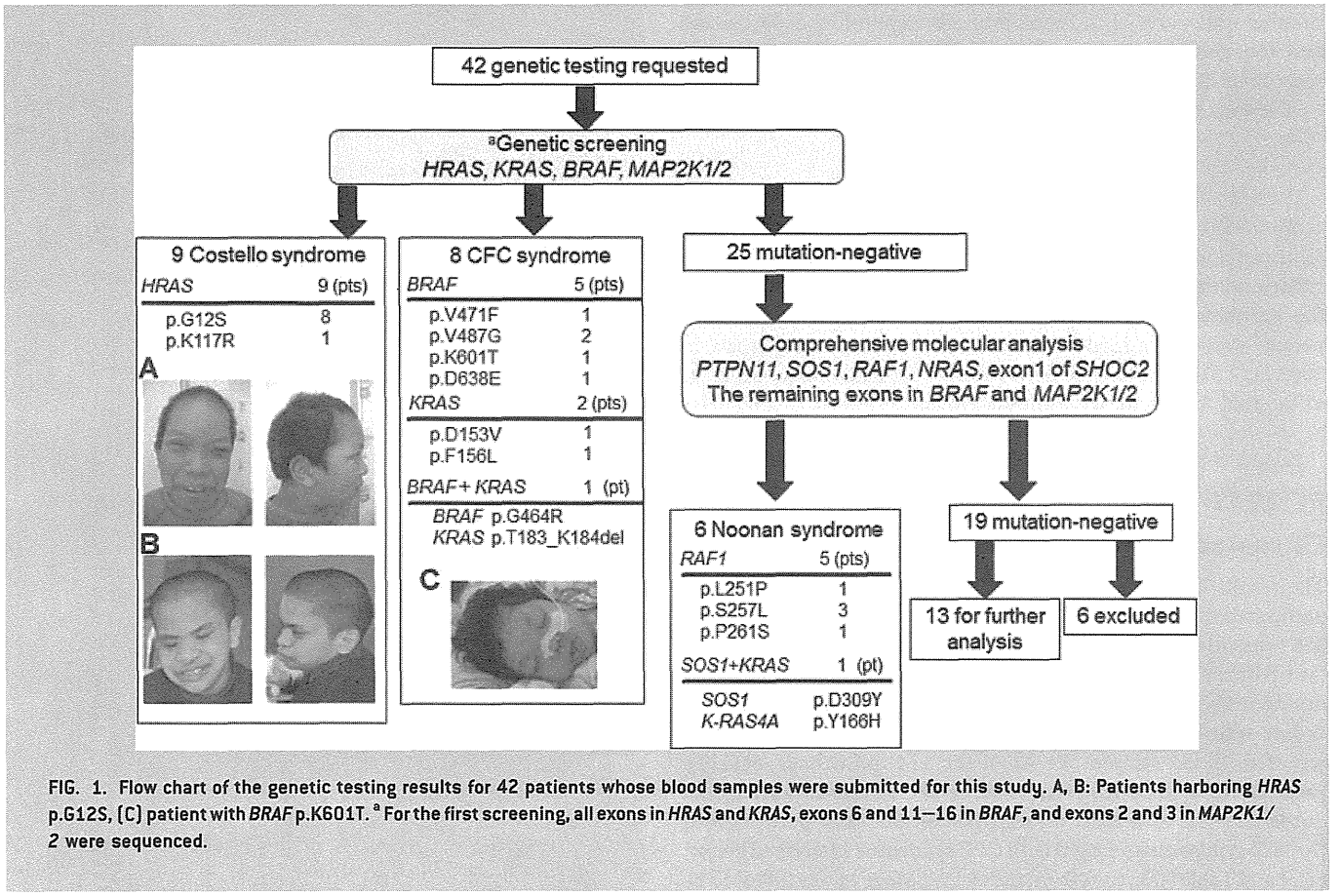

FIG. 1. Flow chart of the genetic testing results for 42 patients whose blood samples were submitted for this study. A, B: Patients harboring *HRAS* p.G12S, (C) patient with *BRAF* p.K601T. [a] For the first screening, all exons in *HRAS* and *KRAS*, exons 6 and 11–16 in *BRAF*, and exons 2 and 3 in *MAP2K1/2* were sequenced.

suspected patients who did not receive the genetic testing. The number of mutation-positive patients estimated by the mutation analysis was calculated using the number of the clinically suspected patients reported in the first-stage survey (PS_k), the ratio of the number of newly identified mutation-positive patients (PD_k), and the total number of patients examined (PA_k). Therefore, the total estimated number of patients in hospitals in stratum k $\sum iN_{ki}$, which responded to the first survey, was calculated as follows:

$$\sum_i iN_{ki} = PR_k + PS_k \frac{PD_k}{PA_k}$$

To calculate the total number of patients in all hospitals listed, we estimated that the mean number of patients among the departments that responded to the survey was equal to that of those departments that did not respond.

The number of patients in stratum k was therefore estimated as

$$\begin{aligned}\hat{\alpha}_k &= \frac{1}{SRT_k RRT_k} \sum_i iN_{ki} \\ &= \frac{1}{\frac{NS_k}{n_k}\frac{N_k}{NS_k}} \sum_i iN_{ki} \\ &= \frac{n_k}{N_k} \sum_i iN_{ki}\end{aligned}$$

where SRT_k, RRT_k, NS_k, n_k, N_k, and N_{ki} denote the sampling rate, the response rate, the number of sampled departments, the total number of departments, the number of responding departments, and the number of departments with i patients in stratum k, respectively.

The total number of patients, $\hat{\alpha}$, was computed as follows:

$$\hat{\alpha} = \sum_k \hat{\alpha}_k$$

The 95% CI of $\hat{\alpha}_k$ was calculated as previously described [Kuriyama et al., 2008]. Five deceased patients with Costello syndrome reported in the first survey (Table I) were excluded in the estimation of prevalence. The prevalence rate per 100,000 people was determined based on the population of Japan in 2009 (127,510,000) with data from the Statistics Bureau, Ministry of Internal Affairs and Communications.

RESULTS

Estimated Number of Patients

The results of the first postal survey and the molecular analysis performed in this study are shown in Table I. Of 1,127 departments, 856 responded to the first-stage survey questionnaire (76%). Fifty-four patients, including five deceased patients, with Costello syndrome with mutations in *HRAS* and 54 patients with CFC syndrome who had mutations in *KRAS*, *BRAF*, or *MAP2K1/2* were reported. Blood samples for 42 of the 114 individuals clinically suspected to have Costello syndrome or CFC syndrome were sent to our laboratory. Molecular screening identified nine patients with Costello syndrome and eight with CFC syndrome (described below, Fig. 1 and Table I). Results from the second-stage survey followed by

TABLE I. Results of the First Postal Survey and the Number of Newly Identified Patients

	Total departments	Surveyed departments	Sampling rate (%)	Departments that responded	Response rate (%)	Reported in the first-stage postal survey			Genetic testing performed	Newly identified	Newly identified
						CS[c] (deceased)	CFCS[c]	CS/CFCS suspected		CS	CFCS
University hospitals	166[b]	163	98.2	158	96.9	11(2)	13	44	15	5	1
Selected hospitals[a]	29	29	100	18	62.1	28(2)	33	16	1	0	1
Institutions for the mentally and physically disabled	208	205	98.6	142	69.3	10(1)	5	16	5	2	1
General hospitals with ≥500 beds	261	254	97.3	205	80.7	5	1	25	12	0	5
General hospitals with 400–499 beds	212	151	71.2	124	82.1	0	0	5	6	2	0
General hospitals with 300–399 beds	402	150	37.3	106	70.7	0	0	5	1	0	0
General hospitals with 200–299 beds	362	70	19.3	43	61.4	0	0	1	1	0	0
General hospitals with 100–199 beds	740	67	9.1	42	62.7	0	2	2	1	0	0
General hospitals with ≤99 beds	830	38	4.6	18	47.4	0	0	0	0	0	0
Total	3210	1127	35.1	856	76	54(5)	54	114	42	9	8

CS, Costello syndrome; CFCS, CFC syndrome.
[a]Hospitals that had asked for genetic testing of Costello/CFC syndrome to our laboratory prior to the survey.
[b]131 university hospitals were listed, and we sent survey forms to 249 physicians in 166 departments.
[c]Possible duplications among patients were excluded.

exclusion of duplicates showed that in total, 63 patients with Costello syndrome and 62 patients with CFC syndrome were identified. Taking into consideration the sampling rates in each stratum of the general hospitals and the number of undiagnosed patients in the clinically suspected patients, we estimated the total numbers of patients in Japan with Costello syndrome and CFC syndrome to be 99 (95% confidence interval, 77 to 120) and 157 (95% confidence interval, 86 to 229), respectively. Therefore, the prevalence of Costello syndrome and CFC syndrome was estimated to be 1 in 1,290,000 (95% confidence interval, 1 in 1,061,000 to 1 in 1,660,000), and 1 in 810,000 (95% confidence interval, 1 in 556,000 to 1 in 1,490,000) individuals, respectively.

Results of the Molecular Analysis

Screening of 42 clinically diagnosed patients identified nine patients with Costello syndrome and eight patients with CFC syndrome (Fig. 1). Eight of the nine patients with *HRAS* mutations had a p.G12S mutation, and the remaining one had a p.K117R mutation. Six of the eight patients with CFC syndrome had *BRAF* mutations (p.G464R, p.V471F, p.K601T, and p.D638E in a single patient, and p.V487G in two patients), and two patients had *KRAS* mutations (p.D153V and p.F156L). One patient had *BRAF* p.G464R, which has previously been reported in a patient with CFC syndrome [Nava et al., 2007], and a novel *KRAS* variation, c.547_552delACCAAG (p.T183_K184del). Parental samples were not available for this patient, and it is unknown if this variation was pathogenic or not. A subsequent, comprehensive mutation analysis showed that *RAF1* mutations, including p.L251P, p.S257L, and p.P261S, were identified in five patients. Four of the five patients had severe perinatal problems, including polyhydramnios, fetal distress, pleural effusion, and hypertrophic cardiomyopathy. An *SOS1* p.D309Y mutation was identified in a single patient diagnosed with Noonan syndrome. The patient also had another novel variation (p.Y166H) in *K-RAS4A*. Her asymptomatic father had the same variation, suggesting that this variation is a benign polymorphism. The five patients with *RAF* mutations and one patient with the *SOS1* mutation were diagnosed as having Noonan syndrome. In the remaining 19 patients who had no mutations, six patients were excluded based on the review of dysmorphologists because of non-matching facial features and clinical manifestations. The remaining 13 patients will be further analyzed.

Clinical-Epidemiological Features of the Patients

We collected detailed clinical-epidemiological information on 43 of 63 Costello syndrome patients and 54 of 62 CFC syndrome patients who were reported in the first postal survey and newly diagnosed by the current study (Table II). Seventeen male and 25 female patients with Costello syndrome and 28 male and 24 female patients with CFC syndrome were reported. Twenty-six of the patients with Costello syndrome [Aoki et al., 2005; Niihori et al., 2011] and 10 of the patients with CFC syndrome [Niihori et al., 2006; Narumi et al., 2008] had been previously studied. Of the Costello syndrome patients, 27 of the 43 patients had *HRAS* p.G12S, five had p.G12A and two had p.G13D, p.G12C, p.G12V, p.G12D, and p.K117R were identified in a single patient. In the patients with CFC syndrome, 38 (70%), eight (15%) and eight (15%) of the 54 patients had *BRAF*, *MAP2K1/2*, and *KRAS* mutations, respectively.

Evaluation of clinical manifestations showed that postnatal failure to thrive and intellectual disability were reported at a rate of more than 95% in both disorders (Table II). Short stature was reported in 72 and 82% of patients with Costello syndrome and CFC syndrome, respectively. The frequency of hypertrophic cardiomyopathy and arrhythmia was significantly higher in patients with Costello syndrome compared to CFC syndrome. In contrast, the frequency of pulmonic stenosis was significantly higher in patients with CFC syndrome compared to Costello syndrome. Abnormal brain structure as detected by CT and/or MRI was reported in eight Costello syndrome patients. Of these eight patients, two were reported as having Arnold–Chiari type I, two had hydrocephalus, one had cortical atrophy, one had hydrocephalus and cortical atrophy, one had tonsillar descent, and one had ventricular dilation and a thinning of the corpus callosum. Abnormal brain structure was also observed in seven CFC patients; two had thinning of the corpus callosum, one had cortical atrophy, one had cortical atrophy, thinning of the corpus callosum and a reduction in white matter volume, one had ventricular dilatation, and one had ventricular dilatation and vermis hypoplasia. Regarding the skin characteristics, the frequency of soft, loose skin and deep palmer/plantar creases was significantly higher in patients with Costello syndrome than in CFC syndrome. Four patients with Costello syndrome developed malignant tumors, including bladder carcinomas, ganglioneuroblastomas and rhabdomyosarcomas. Two patients with CFC syndrome were previously reported as developing ALL and non-Hodgkin lymphoma [Makita et al., 2007; Ohtake et al., 2011]. Five patients with Costello syndrome were deceased. Two patients died from ganglioneuroblastoma and rhabdomyosarcoma. One patient died from tachycardia-induced cardiomyopathy at age 18 months.

The age distribution of the 38 patients with Costello syndrome and the 53 CFC syndrome patients whose ages were reported in the second-stage survey is shown in Figure 2. There were major peaks at 5 years of age in both diseases. The oldest patient diagnosed with Costello syndrome was 22 years of age, while the oldest patient with CFC syndrome was 32 years. Six patients with Costello syndrome and nine patients with CFC syndrome age 18–32 years were identified (Table III). Analysis of their daily living activities showed that 10 individuals could walk independently, one had an abnormal gait, one had a cane-assisted gait, and one used a wheelchair. Two patients with *BRAF* mutations were bedridden. All patients showed intellectual disability, and eight (severe in three patients with Costello syndrome and three patients with CFC syndrome, very severe in two patients with CFC syndrome) were severely disabled. Daily conversation was possible for three individuals. Simple conversations and two-word sentences were possible for four and three patients, respectively. Eleven patients lived at home. Three individuals had graduated from a school or public school for disabled children. Eight adults worked in vocational training facilities. Thirteen patients were able to feed themselves, but two of them sometimes needed assistance with feeding. Two patients with CFC syndrome were bedridden and needed full assistance with feeding and toileting.