Original Research Article

A Novel Multidisciplinary Approach Toward a Better Understanding of Cranial Suture Closure: The First Evidence of Genetic Effects in Adulthood

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ABSTRACT: Objectives: The primary objective of this study was to perform new, relevant information about cranial suture closure in adults. Single nucleotide polymorphisms (SNPs) in targeted genes were examined, which encode factors that play an important role in cranial suture development and maintenance. Our hypothesis was that some of these genes and polymorphisms can influence the cranial suture obliteration status in adulthood as well.

Methods: Ossification of cranial sutures was ascertained according to Meindl and Lovejoy's vault system (1985: Am J Phys Anthropol 68(1):57–66), and peripheral blood samples were collected during autopsy procedure of 106 individuals at the Department of Forensic and Insurance Medicine, Semmelweis University, Hungary. Genotyping of SNPs was conducted using competitive allele-specific polymerase chain reaction KASPar chemistry. Multivariate linear models were used to test whether SNP polymorphism of the investigated genes has a significant effect on the ectocranial suture synostosis in adults.

Results: The msh homeobox 1 (MSX1): rs3821947 polymorphism showed significant association with the extent of suture obliteration.

Conclusions: Cranial suture closure in adults is a complex, multifactorial process. According to previous results MSX1 has a role in calvarial bone development and it has an effect on sutural mesenchyme in latter postnatal stages. Our results demonstrate MSX1 effects on suture obliteration in adulthood. These findings represent new, relevant information indicating that genetic background can have an impact on cranial suture closure in adults. Am. J. Hum. Biol. 25:835–843, 2013.

Researchers have taken a keen interest in the phenomenon of cranial suture synostosis. During normal development cranial sutures remain open to support bone growth in parallel with brain expansion, and theoretically cranial expansion ends with the fusion of bone plates creating the neurocranium somewhere in the third decade of life (Opperman, 2000). In practice, however, this simple formula does not always follow the route that nature and human understanding have prescribed for it. Disturbances during embryogenesis and early postnatal life in morphogenesis of the bones of the cranial vault and suture patency can cause severe disfigurement, mental retardation or even death. Suture obliteration in adulthood does not have such dire consequences, but it can influence the shape and stability of the skull, and cranial suture closure in adults has always been one of the main interests of anthropologists. Observation of the whole process and findings from different fields could produce new answers and give rise to new questions.

ORIGIN OF CRANIAL SUTURE

Morphogenesis of the vertebrate skull is a long, complex developmental process. Anatomically the human skull consists of two elements: the neurocranium, which protects the brain, and the viscerocranium, which is formed by the bones composing the face and covering the pharynx, the oral cavity and other parts of the upper respiratory tract. With the exception of the mandible, all elements of the skull are connected by connective tissue sutures.

The neurocranium and viscerocranium, can be derived from different parts ontogenetically (Carlson, 2009). Ossification of the cranial bones can occur in two different ways. Elements of the basicranium (occipital, sphenoidal, partly temporal, and deep overlying elements of nasal cavity) follow the process of endochondral ossification, whereas the remaining bones undergo intramembranous ossification, through direct ossification of mesenchyme and remain separate and connect each other by connective tissue sutures. During normal postnatal development, some sutures remain open and some of them close. This depends on the fine cooperation of several molecules. Differences in origin can be particularly important during the observation of the development of different structures.

Suture formation seems to be initiated by growth factor signalization of the approximating osteogenic fronts (Opperman et al., 1993; Roth et al., 1996). As the bone edges near one another on the same level, end-to-end sutures can develop between them in the midline of the cranial vault and at the sites of overlapping bone plates that approach each other on different levels overlapping sutures may appear (Furtwangler et al., 1985; Johansen and Hall, 1982).

GENES AND MECHANISMS

Not only the formation of the calvarial sutures but also their later behavior depends on the impact of several

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molecules and signaling pathways. To understand the nature of these mechanism two important target pools must be observed: craniosynostosis cases and vertebrate models.

Craniosynostosis is the premature closure of the cranial sutures, which can be isolated, affecting only the sutures of the cranial vault, or part of a syndrome. The prevalence of craniosynostosis is ~1 in 2,100–2,500 births (Boulet et al., 2008; Cohen, 2000a,b; Hehr and Muenke, 1999; Lajeunie et al., 1995, 1996), which makes it a fairly common pediatric anomaly. The first gene that was found to be in association with craniosynostosis was the msh homeobox 2 (MSX2) gene, a mutation that causes the Boston-type craniosynostosis (Jabs et al., 1993). In subsequent years more mutations were discovered in relation to syndromic craniosynostosis cases in the fibroblast growth factor receptor (FGFR) genes (Bellus et al., 1996; Jabs et al., 1994; Muenke et al., 1994, 1997; Reardon et al., 1994). At this point, more than 180 craniosynostoses and more than 60 different mutations in syndromic cases have been identified, the majority of which happen in the FGFR2 gene. Most of the major forms of craniosynostoses have an autosomal dominant inheritance, such as mutations in FGFR2 in Apert and Crouzon syndrome, in FGFR1 and/or in FGFR2 in Pfeiffer syndrome, in FGFR3 in Muenke syndrome, in the TWIST gene in Saethre-chotzen syndrome, and in fibrillin 1 in Marfan syndrome, but there are some exceptions, for example the X-linked craniofrontonasal syndrome caused by the mutations in the ephrin B1 gene (Agochukwu et al., 2012; Wilkie, 1997). The mutations themselves are mostly missenses, such as in the case of FGFRs, but in the TWIST gene most anomalies are caused by nonsense changes and 21 bp duplications and complete deletions (Johnson et al., 1998; Kress et al., 2006; Wilkie, 1997). However, missense substitutions have been found in the TWIST protein in sagittal synostotic cases (Bialek et al., 2004; Kress et al., 2006), which suggests that these genes have an important role not only in syndromic craniosynostoses, and might have an influence on nonsyndromic cases and in later ossification during normal development as well. Testing sporadic, nonsyndromic cases seems to be a much greater challenge, while in most individuals with sagittal, metopic, or lambdoid synostoses—with the occasional exception of some—the efficiency of the known genetic diagnoses was close to zero (Wilkie et al., 2007). There are a few exceptions in coronal synostoses in which some single-gene mutations have been found in the background, something that never occurred in any of other suture synostoses. The different behavior of coronal sutures is probably caused by the fact that they lie at the boundary between two different embryonic tissues (Wilkie et al., 2010). These facts and possible explanations suggest that the majority of craniosynostoseses are due to multifactorial inheritance.

In addition to genome-wide linkage analyses and candidate gene studies in affected families, vertebrate models have an extremely important role in research on the nature of cranial sutures. According to rat and mouse modeling systems, suture morphology is determined by at least three different tissues, the sutural mesenchyme, the osteogenic fronts and the dura mater (Kim et al., 1998). Signals from the dura mater are more important prenatally and dominance in suture patency regulation shifts to the osteogenic fronts postnatally (Kim et al., 1998). Any disturbances in any element of the signaling pathways can cause conditions, sometimes even very severe, and many of these factors together with the environmental effects probably influence the cranial structure throughout life. The expression of different factors is highly variable according to the developmental status. Expression also varies depending on different tissues and even different sites of the cranial elements. Before suture formation, bone morphogenetic protein 4 (BMP4), BMP7, FGF9, MSX1, MSX2, and TWIST factors are present almost everywhere in epitheliomesenchymal signaling, while FGFRs are expressed only in the approaching bone fronts, transforming growth factors β (TGF-βs) in the osteogenic fronts and the underlying dura (Opperman, 2000). When bone fronts are closing and overlapping, sonic hedgehog (SHH), patched (PTC), and inhibitor of DNA binding (ID) are expressed in regulation of pattern formation, whereas TWIST and MSX2 are absent. This is not surprising, as the dominant negative helix–loop–helix Id, upregulated by BMP2, inhibits basic helix–loop–helix transcription factor Twist (Rice et al., 2000). Later it was found that not only the presence of Twist1 but even its dimer partner defines its activity in suture maintenance (Connerney et al., 2006, 2008) and can have both positive and negative effects on mesenchymal cells (Connerney et al., 2006). CBFA1, osteopontin, osteonectin, alkaline phosphatase and bone sialoprotein (BSP) in the bone and type I and III collagens with FGFR 1–3 and TGF-β 1–3 are expressed in the bone fronts, while suture matrix expresses type III collagen, FGF9, MSX1, and FGFR1 (Opperman, 2000). During suture fusion, factors, which play a role in bone formation, are present in the suture matrix, such as type I collagen, TGF-β 1–2, FGFR 1–2, BSP-I, and CBFA1, fibroblast growth factor 2 (FGF-2), and insulin-like growth factor 1 (IGF-1) in the bone fronts, while MSX1, ID, SHH, PTC, and FGF-9 are downregulated (Opperman, 2000). In a study of calvarial development in mice and the use of an in vitro modeling system, BMP2 and BMP4 were found to be expressed in the osteogenic fronts, the latter with MSX1 and MSX2 in the sutural mesenchyme as well. As MSX1 and MSX2 homeobox-containing transcription factor are targets of BMP and FGF signaling (Satokata and Maas, 1994), locally applied BMP4 proteins influenced the expression of MSX1 and MSX2, which led to an increase in tissue mass (Kim et al., 1998). Kim and colleagues suggested that BMPs play an important role in osteogenic cell differentiation, while the cooperation of MSX proteins and FGFs affect mature osteoblasts later. In postnatal stages the expression of FGF4 increased and induced the expression of MSX1. Postnatally BMP4, FGFR2, SHH, and PTC expression was found in a patched pattern in the osteogenic fronts, and Kim et al. (1998) suggested that these factors interact with each other through a PTC-dependent pathway to prevent early suture obliteration, and suture patency after birth might be controlled by signaling events at the osteogenic fronts, where SHH plays an important role. They also highlight the fact that different signaling pathways are involved in cranial suture development in pre- and postnatal stages. One of the most essential examples of the signalization hierarchy, is the fact that BMPs are expressed along the whole cranium during fetal bone morphogenesis, inducing general bone formation, and BMP antagonist noggin is expressed on the sites of cranial sutures. Locally expressed FGF-2 downregulates noggin, and BMPs can

take control of the fate of cranial sutures (Warren et al., 2003). Start here work with animal models demonstrated that the possible effects of increased BMP2 or its receptor can even cause premature suture fusion (Dwivedi et al., 2013; Kinsella et al., 2011; Komatsu et al., 2013). Furthermore tensional forces in the dura mater were also observed as a consequence of rapid growth of the neurocranium influence suture patency, as well as an increase in expression of FGFR 1 and 2 (Ogle et al., 2004).

CRANIAL SUTURE CLOSURE IN ADULTHOOD: AGE RELATION HYPOTHESIS

In the last century many skeletal age estimation methods were developed on the assumption that suture closure is the part of the aging process. Cranial sutures became one of the primary tools and interests of the anthropologists (Todd, 1924; Todd and Lyon, 1925). According to some authors, both ecto- and endocranial closure seemed to correlate with age, and individual techniques based only on suture closure or obliteration as a part of a complex age estimation method have been established. Some authors, such as Acsádi and Nemeskéri, use endocranial suture closure as a part of their complex age estimation method (Acsádi and Nemeskéri, 1970), and Lovejoy et al. (1985) use ectocranial closure as one of their five indicators. However, the applicability of these methods has been a manner of dispute. Some authors found better correlation between endocranial suture obliteration and age (Galera et al., 1998; Key et al., 1994; Todd and Lyon, 1925; Wolff et al., 2012), while Meindl and Lovejoy (1985) suggest that their ectocranial suture closure method is more appropriate, especially in older individuals. Although the people who developed these methods recommend their techniques for general use in age estimation, several authors have questioned their efficiency in practice, and contradictory results were reached in the pursuance of independent tests on known sex and age populations all around the world. In addition to contradictions in usage, reproducibility and efficiency, some researchers even found relevant sexual dimorphism in the range of cranial suture closure (Key et al., 1994; Sahni et al., 2005; Singer, 1953), while others observed no differences between genders (Acsádi and Nemeskéri, 1970; Hrdlicka, 1952; Perizonius, 1984; Todd, 1924; Wolff et al., 2012). Despite some attempts to refine and correct cranial suture aging methods (Galera et al., 1998; Key et al., 1994; Perizonius, 1984), all have limitations, suggesting not only relevant interpopulation difference in the pattern of suture obliteration, but understandably throw into question the fundamental premises of these practices (Brooks, 1955; Cray et al., 2011b; Hershkovitz et al., 1997; Powers, 1962; Sahni et al., 2005; Singer, 1953; Wolff et al., 2012). One of the more innovative methods available is Nawrocki's (1998) assessment of suture obliteration as an indicator of age at death, but it still suffers from the same problems inherent in adult skeletal age estimation. Some other morphological methods or revisions of these methods showed similar or slightly better application in age estimation, but authors still warrant the practitioners in the usage of their techniques when applied on different populations (Iscan et al., 1987; Katz and Suchey, 1989; Osborne et al., 2004; Russell et al., 1993; Sahni et al., 2005; Wolff et al., 2012; Yavuz et al., 1998).

CRANIAL SUTURE CLOSURE IN ADULTHOOD: MASTICATORY STRAIN HYPOTHESIS

Another potential mechanism that can influence cranial suture patency and closure is the mechanical loads of the masticatory muscles. Fibrous sutures are under tension because of the growing brain (Moss, 1969; Moss and Young, 1960), and according to some research under the tension and compression forces of mastication. Some authors suggest that these cyclic strains have a high impact on suture growth, forming and closure, especially on ectocranial sites (Byron et al., 2004, 2006; Herring, 2008). During the examination of midline sutures in pigs, Sun et al. (2004) found increased ectocranial interparietal suture strain with age and decreased forces on closed sutures. They suggest that fusion can be the result of rapid bone apposition occasionally leading to obliteration on the ectocranial suture sites, and it can be partly initiated by mechanical factors (Sun et al., 2004). However, Kanisius and Luke (1994) affirm that morphology of the midline sagittal suture in humans is not influenced by mechanical forces, because its location neutralizes it from muscle size and activity. Cray et al. (20011a, 2011b) found no strong association between skull size and suture activity in *Pan* and *Gorilla*, but found statistically significant relationships between rate of obliteration and dental status. This phenomenon occurred only after neurocranial expansion, so they suggest that the primary role in early suture formation is played by the expanding brain with biomechanical adaptation affecting suture obliteration in later periods dependent on masticatory habits and activity (Cray et al., 2011b). While studying Aleutian Island human remains, Cray et al. (2011b) found no correlation between cranial shape and suture synostosis and assumed that these patterns may be population dependent. The latter conclusion and similar findings of Kanisius and Luke (1994) allude to probable genetic predisposition in suture patency and closure. This had already been highlighted in the work of Hershkovitz et al. (1997) but could not be supported given the lack of relevant information. On the other hand, it seems that most of the factors that are important in morphogenesis and maintenance of cranial sutures increase under stress and tensile forces (Cray et al., 2011a). In conclusion, cranial shape may well be the result of cranial growth influenced by the expansion of the brain, the timing of cranial suture closure, and genetic factors, and there may also be relevant epigenetic factors, such as masticatory muscles, diet, laying habits in early childhood and metabolism.

METABOLIC DIFFERENCES

Finally changes in metabolism (e.g., rickets) can cause secondary craniosynostosis, which is a quite well-known phenomenon for clinical practitioners (Boulet et al., 2008; Currarino, 2007; Garg et al., 2010; Inman et al., 2008; Murthy, 2009; Reilly et al., 1964; Shetty et al., 1998; Stickler et al., 1970). One of the key molecules in this association seems to be the FGF-23, a phosphate-regulating protein, encoded on a gene in 12p13 that influences bone mineralization and its elevated levels can cause craniosynostosis due to a cross-binding with FGFR2 and 3 at cranial sutures (Murthy, 2009; Saito et al., 2003; Yamashita et al., 2002). One possible condition what indirectly regulates FGF-23 synthesis happens in X-linked

hypophosphotemic rickets (Currarino, 2007; Murthy, 2009), but some other associations with hypophosphotemic bone diseases and craniosynostosis have been reported as well (Shetty et al., 1998). These findings suggest that small differences in individuals' metabolism can lead to interactions between different signalization pathways and can cause differences in the behavior of other organ systems as well.

PURPOSE OF THE STUDY

First the authors presented an overview of cranial sutures and information that can be interesting to different areas of research that deal with cranial sutures. These observations led the authors of this article to the conclusion that cranial suture closure is a multifactorial phenomenon where age, environment, lifestyle, diet, muscular robustucity and activity, and pathological conditions might be among the several factors that influence suture maintenance. Above all, genetic background, such as inheritable expression patterns of molecular factors that influence suture growth and patency can be a significant component of this complex process of regulation. The purpose of this study was to examine some single nucleotide polymorphisms (SNPs) in targeted genes which encode factors that play an important role in cranial suture development and maintenance in relation to cranial suture obliteration status. The assumption was that if some alterations in relevant genes can cause severe cranial manifestations in childhood, some of them can influence suture patency in later postnatal stages as well. To follow up on our earlier research (Wolff et al., 2012), our primary objective was to provide a novel explanation of cranial suture closure in adults by estimating age on the same sample from which the genetic data were drawn.

MATERIALS AND METHODS

DNA and cranial suture closure data was obtained from 106 individuals during autopsies at the Department of Forensic and Insurance Medicine, Semmelweis University, Hungary. All study subjects belonged to the Hungarian (Caucasian) population. The research was approved by the Ethics Committee of the Hungarian Medical Research Council (ETT TUKEB) in accordance with the tenets of the Declaration of Helsinki. Actual ages were furnished by the police (according their ID cards, official documents, etc.) and were not known at the time of the age estimation procedures. All individuals under investigation were Hungarian citizens, and the information concerning their identities was accurate. Unidentified individuals were excluded from the study.

Cranial suture closure

The assessment of the extent of cranial suture closure has been prepared according to Meindl and Lovejoy's vault technique (Meindl and Lovejoy, 1985) because this method observes only the closure of main sutures, namely the lambdoid, sagittal, and coronal suture sites and describes suture obliteration on a relatively large range. These options were the main interest of the research. In the case of each individual, seven vault landmarks were scored on a scale from zero to three—0 = completely open, 1 = 1–50% fusion, 2 = 51–99% fusion, and 3 = complete fusion—based on the amount of obliteration. These scores were summed for each cranium, producing a final score from 0 to 21. On bilateral suture sites, where closures were in different phases, scores were averaged for further calculations. Cadavers with further trauma or pathological changes, where the observation or the fusion of the sutures could have been influenced, were not included in the investigation. All the suture closure examinations were taken by the first author and no significant intraobserver error was found at the assessment (paired t test; $P = 0.286$).

Laboratory methods, genotyping

Genomic DNA from adults during medicolegal autopsies was obtained from whole, peripheral blood using the Chelex extraction process (Bio-Rad Laboratories, Munich, Germany) at the DNA Laboratory of the Department of Forensic and Insurance Medicine. Genotyping of SNPs was conducted using competitive allele-specific polymerase chain reaction (PCR) KASPar chemistry (KBiosciences, Hertfordshire, UK) according to the manufacturer's instructions at the Department of Genetics, Cell- and Immunobiology, Semmelweis University. Only SNPs that have a genotyping call rate over 90% were included in the analysis.

Candidate gene selection

From the scientific literature we selected six candidate genes which, according to earlier findings, seemed to be relevant in cranial suture formation and/or cranial suture synostosis and might have an effect on suture closure in adults. We searched online databases for SNPs for these genes (NCBI, Genecards). Twelve SNPs were selected altogether. The selection criterion was: minor allele frequency >10%. Sources of data were HapMap and USCS genome databases. The selected SNPs and additional information can be seen in Table 1.

Statistical analysis

Allele frequencies were calculated by allele counting. Statistical analyses were carried out with R 2.14.0 software (Fox, 2005; R, 2005). Kendall's correlation was used to test the expected correlation between the real age and the state of ectocranial suture closure. Inaccuracy and bias were defined as the average absolute error of age estimation and the mean over and under prediction. One-way analysis of variance (ANOVA) model and Tukey's post hoc pairwise tests were used to find significant differences between neighboring stages. We applied analysis of covariance models in a general linear model framework to test whether SNP polymorphism of the investigated genes has a significant effect on the ectocranial suture synostosis of adults. The scores of suture closure (Meindl and Lovejoy, 1985) were treated as a response variable, while known age and certain SNP polymorphisms were considered explanatory variables. The criteria of general linear models (i.e., the linearity of the regression, the normality of the residuals, no heteroscedastic pattern in the standard deviation of the residuals, absence of influential data points) were satisfied according to diagnostic plots (e.g., scale-location plot, quantile-comparison plot, and Cook's distance plot). All reported P values are two tailed.

RESULTS

Five individuals were omitted from the research due to poor DNA quality (three samples), unknown age at the

TABLE 1. Description of selected single nucleotide polimorphisms

SNP rs	SNP position	Alleles	Gene	Role	Amino acid change	Minor allele	Minor allele frequency
rs17563	chr14: 53487272	A/G	BMP4	Missense	Alanine/valine	A	0.394
rs2761887	chr14: 53494802	C/A	BMP4	Promoter	–	C	0.469
rs4898820	chr14: 53496807	T/G	BMP4	Promoter	–	G	0.485
rs1061237	chr17: 45617774	A/G	COL1A1	3′UTR	–	G	0.314
rs1061947	chr17: 45617118	G/A	COL1A1	3′UTR	–	A	0.159
rs308393	chr4: 123966392	A/C	FGF2	Promoter	–	C	0.173
rs308395	chr4: 123966069	C/G	FGF2	Promoter	–	G	0.138
rs1047111	chr10: 123347551	C/T	FGFR2	5′UTR	–	C	0.181
rs3135715	chr10: 123344716	T/G	FGFR2	Promoter	–	G	0.235
rs1907998	chr4: 4907480	A/G	MSX1	Promoter	–	G	0.323
rs3821947	chr4: 4911039	A/G	MSX1	Promoter	–	G	0.434
rs288746	chr7: 155299433	G/A	SHH	Promoter	–	G	0.168

TABLE 2. Age distribution

Age intervals	Males (*N*)	Females (*N*)
18–30	6 (10.3%)	3 (7.0%)
31–40	4 (6.9%)	3 (7.0%)
41–50	5 (8.6%)	0 (0.0%)
51–60	11 (19.0%)	10 (23.2%)
61–70	12 (20.7%)	7 (16.3%)
71–80	9 (15.5%)	7 (16.3%)
81–100	11 (19.0%)	13 (30.2%)
Total	58 (100.0%)	43 (100.0%)
Age range	20–91	18–97

TABLE 3. Descriptive statistics according to age groups

	Hungarian sample ($N = 101$)				
Age intervals	Mean age	SD	Median	95% confidence interval	Range
17–30	24.4	4.4	26	18.4–29.0	18–29
31–40	35.3	3.3	34	31.3–39.85	31–40
41–50	44.4	3.6	43	41.1–49.6	41–50
51–60	55.3	3.2	55	51.0–59.5	51–60
61–70	66.2	2.5	67	61.45–69.55	61–70
71–80	75.75	3.3	76	71.38–80.0	71–80
81–100	87.7	4.4	87.5	81.58–97.0	81–97

time of death (one sample), or pathological alterations on the skull (one sample). Finally, 101 individuals were involved to the study. The characteristics of the study population are shown in Table 2. All age intervals were represented in males, but there was a lack of samples in the 41–50 age category in females, and most of the individuals belonged to the 51–100 age range. Table 3 shows the main descriptive statistics and 95% confidence intervals of the mean age for the whole sample by age groups.

Cranial suture closure: age estimation

Table 4 shows the main descriptive statistics and 95% confidence intervals of the mean age for all phases in the Hungarian sample. The original data on which the Meindl and Lovejoy vault technique was based are also represented in Table 4. As we have already observed regarding this Hungarian population (Wolff et al., 2012), mean ages did not increase with the progression of suture closure, and 95% confident intervals overlap to a great extent. Standard deviations are always above 10 years and 95% confidence intervals were greater than 30 years, even in the less variable Phase 1. Kendall's correlation was performed to test the correlation between known age and the extent of obliteration and to estimate the variance in age explained by obliteration. Significant positive correlation was found ($N = 101$; $P < 0.001$), but associated with a low correlation coefficient ($\tau = 0.282$). The slope of the regression lines did not differ significantly between genders ($P = 0.823$) in a linear model, thus the two genders could be treated together allowing for larger samples for statistical analysis. Between most neighboring phases a great overlap of age distribution was observed and a significant difference was found only between Phases 2 and 3 using a one-way ANOVA model and Tukey's post hoc pairwise test ($P < 0.001$). Table 5 shows the average inaccuracy and bias for cranial suture closing by age groups. Bias shows that the technique overages adults under 30 and

TABLE 4. Descriptive statistics in ectocranial suture closure observation

		Hungarian sample ($N = 101$)					
Phase	Closure score	*N*	Mean age	SD	Median	95% confidence interval	Range
1	0	4	36.0	15.51	33.5	21.45–54.8	21–56
2	1–2	9	33.78	18.28	29.0	18.4–66.2	18–67
3	3–6	40	65.18	15.49	65.0	35.95–87.1	34–91
4	7–11	24	65.46	16.94	65.5	31.86–97.0	29–97
5	12–15	16	76.0	13.59	78.0	48.75–91.0	39–91
6	16–18	7	75.71	23.46	88.0	35.85–92.55	33–93
7	19–20	1	52	NA	52	NA	NA

underages them over 40 years of age. Estimation was most accurate between 31 and 40 years of age. Inaccuracy ranged from 6.2 to as high as 47.06 years for the oldest individuals. Altogether, the chronological age was predictable only in 24% of the individuals.

Cranial suture synostosis: genetic background

Among the investigated genes and loci, only the rs3821947 SNP polymorphism of the MSX1 gene had a robust significant effect on ectocranial suture syostosis, while with the exception of known age none of the other examined SNPs had significant explanatory power in the other genes' models.

In the case of MSX1 rs3821947 polymorphisms, both known age (slope = 0.10, $P < 0.001$) and genotypes ($P = 0.012$) affected ectocranial synostosis significantly ($n = 101$, $df = 97$, adjusted $R^2 = 0.22$, $F = 10.3$). The fact that both these explanatory variables were significant predictors suggests that they explained unique variance in cranial suture score. Neither gender ($P = 0.267$) nor the interactions between explanatory variables ($P = 0.105$–0.223) were significant predictors of cranial suture score.

TABLE 5. Bias and inaccuracy in ectocranial suture closure

Age intervals	Ectocranial suture closure
17–30	
Bias	6.16
Inaccuracy	6.27
31–40	
Bias	1.83
Inaccuracy	6.20
41–50	
Bias	−7.82
Inaccuracy	7.82
51–60	
Bias	−17.76
Inaccuracy	17.76
61–70	
Bias	−26.30
Inaccuracy	26.30
71–80	
Bias	−36.30
Inaccuracy	36.30
81–100	
Bias	−47.06
Inaccuracy	47.06

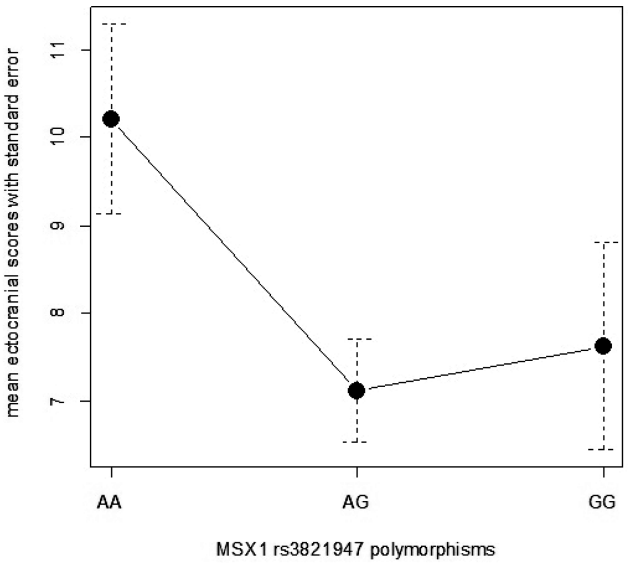

Fig. 1. Mean and standard error of ectocranial scores by MSX1 rs3821947 SNP groups.

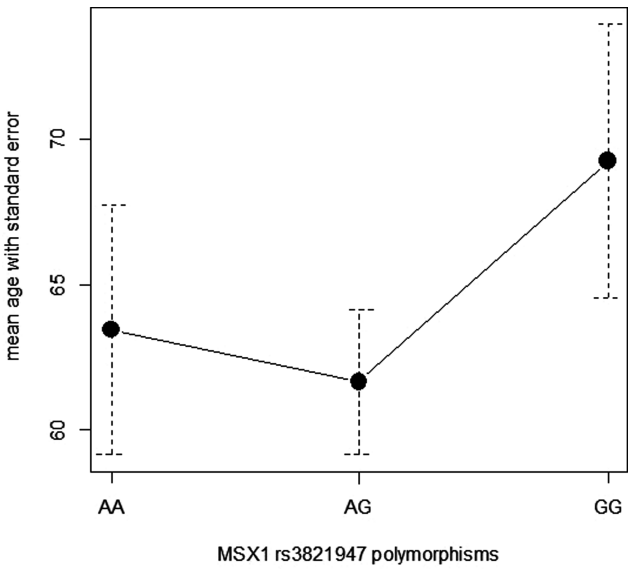

Fig. 2. Mean and standard error of known age by MSX1 rs3821947 SNP groups.

This relationship was not driven by sampling bias (i.e., a given polymorphism group has higher ectocranial scores only because older people belong to that group), as the highest average ectocranial score was in the rs3821947AA genotype group (Fig. 1), while the highest average age was in the rs3821947GG genotype (Figs. 2 and 3).

DISCUSSION

The oldest and probably most controversial methods in skeletal age estimation are those based on the obliteration of cranial sutures. Several studies have investigated the reliability of these systems, and several revised methods have been used on different populations (Key et al., 1994; Perizonius, 1984) in the last decades, but their accuracy in overall use is still problematic. Moreover, many researchers have observed that ectocranial suture closure pattern in particular has an extreme variability, leading them to conclude that the methods based on them are inaccurate and problematic in determination of the age of skeletonized individuals (Brooks, 1955; Cray et al., 2011b; Hershkovitz et al., 1997; Powers, 1962; Sahni et al., 2005; Singer, 1953; Wolff et al., 2012). Despite the high variability in the usage of the method, it is still in practice even in forensic investigations (Garvin and Passalacqua, 2012), although these techniques are not the most popular ones. One cannot deny the temptation to use cranial sutures as a basis for age determination, especially under bad preservation circumstances, when other, more fragile age estimation sites are unobservable. Given the clear limitations associated with the estimation of age at death in the adult skeleton using cranial suture obliteration caution is warranted.

The use of cranial sutures as an indicator of age at death in the adult skeleton has improved our understanding of suture maintenance and obliteration, but it is still unclear why so much variation in suture closure exists across the adult lifespan. There is much diversity in skull morphology due to genetics, metabolism, diet and sleeping environment early in life. In addition to the popular assumption that masticatory strain has a strong influence on cranial suture patency, others have reported the potential importance of genetic components in suture closure as well (Cohen, 1993; Cray et al., 2011a; Hershkovitz et al., 1997). Meanwhile, while there is an enormous literature on the genetic background of early suture closure in childhood, there is a lack of similar studies in adults. This is understandable from the medical perspective when the consequences of suture obliteration are taken into consideration, but it should not be forgotten that the more we know about the behavior of sutures, the better we can understand and manage the mechanisms under different circumstances. The other difficulty with research on adult craniosynostoses is that most examinations cannot be carried out in vivo in humans, for example measurement of real-time gene expression and levels of RNA synthesis in different sites of cranial sutures is almost impossible and raises ethical issues, and normally the sutures of the most

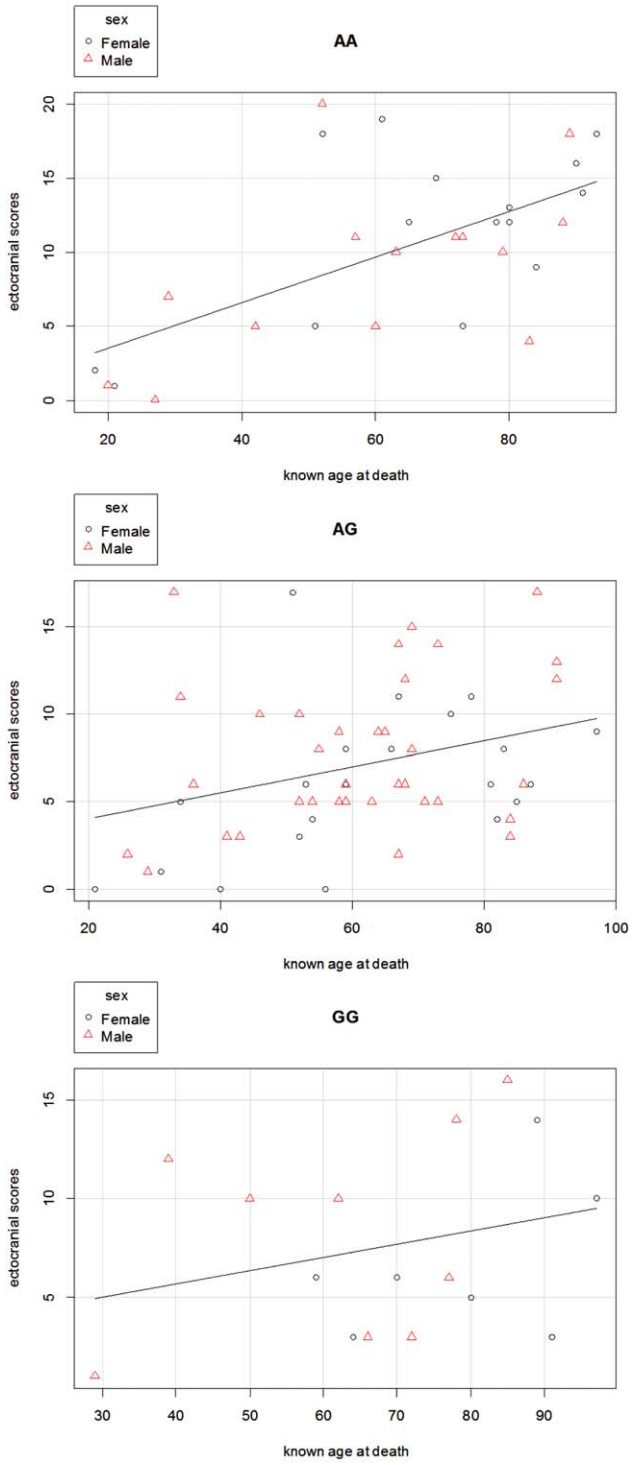

Fig. 3. Relationship between mean age and suture closure score by genotypes. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

popular animal models do not close, even in postnatal stages (except the interfrontal suture in rodents, Mehrara et al., 1999). These conditions and long-standing problems and issues prompted the authors to adopt this multidisciplinary approach. We offer a possible explanation for a phenomenon that is relevant primarily to the field of forensic anthropology, but can be interesting to any area of research that deals with cranial sutures.

MSX1 transcription factor is a member of the muscle segment homeobox family, and it plays an important role in limb-pattern formation and craniofacial development, especially in odontogenesis, mouth structure formation and tumor growth inhibition according to current scientific knowledge. The gene is located on the short arm of Chromosome 4. The function of MSX1 in cranial suture synostosis is not clear yet, but some studies have detected the expression and proved its attendance at cranial suture sites (Kim et al., 1998; Opperman, 2000). Although no mutation associated with early craniosynostoses was found in the MSX1 gene, it represents a fundamental element of the signaling pathways of cranial bone and suture development and maintenance parallel to MSX2. Both are targets of BMP signalization, while FGFs only influence the transcription of MSX1, and it was expressed in the suture mesenchyme and in the dura mater continuously in postnatal stages as well (Kim et al., 1998). Some authors suggest that postnatal suture patency might be regulated by the signaling events of the osteogenic fronts, and SHH plays an important role in here in interaction with the other signaling pathways (Kim et al., 1998).

Furthermore, a weak association between BMP4 rs2761887 polymorphism and cranial suture closure was found; however, the results were not entirely convincing, so the discussion of this finding was omitted from the results. But taking into consideration that gene expression studies in animal models revealed that both MSX1 and BMP4 proteins are expressed in postnatal stages, and that these two genes are on the same signaling pathway, these results strongly suggest the possible effect of these mechanisms on cranial suture closure in adults. Therefore, further investigations are recommended on independent, larger populations.

CONCLUSIONS

In conclusion, the authors provided a new perspective on cranial suture closure in adulthood. Practitioners have long been aware of the limitations associated with the use of cranial sutures as an indicator of age at death in the skeleton; however, this is not unique, but rather a general problem shared by all methods of age estimation in the adult skeleton. The large, diverse literature dealing with cranial sutures suggests that the closing of cranial vault sutures might be a much more complex, multifactorial phenomenon, where chronological age is only one indicator among many, taking into consideration the possibility that the more time that has elapsed, the more factors may influence their attendance. The authors focused on the possible effects of the genetic background and found significant correlation with MSX1 rs3821947 polymorphisms in relation to cranial suture obliteration status.

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